Inflammatory Resolution: The Role of Cyclopentenone Prostaglandins, Adenosine and Lymphocyte Trafficking

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Table of contents

Index of Figures 8

Index of Tables 10

Acknowledgments 11

Declarations 12

Publications from this thesis 13

Abstract 14

Chapter 1 Introduction 16

1.1 Overview of Inflammation 17

1.1.1 The necessity of the inflammatory response 17

1.1.2 The transition from acute inflammation to resolution 18

1.1.3 Wound Healing 22

1.2 Apoptosis and Phagocytosis 23

1.2.1 Apoptosis 23

1.2.2 Phagocytosis 24

1.3 Innate versus Adaptive Immunity 25

1.3.1 Innate Immunity 25

1.3.2 Adaptive immunity 27

1.4 Cellular components in inflammation and resolution 29

1.4.1 Granulocytes 29

1.4.1.1 Mast cells 29

1.4.1.2 Polymorphonuclear neutrophils 29

1.4.2 Mononuclear cells 31

1.4.2.1 Monocytes and macrophages 31

1.4.2.2 Dendritic cells 33

1.4.2.3 Lymphocytes 34
1.4.2.3.1 T cells 35
1.4.2.3.2 B-lymphocytes 36
1.5 Mediators of resolution 38
1.5.1 Arachidonic acid metabolism by cyclo-oxygenase 38
1.5.1.1 COX and inflammation 42
1.5.1.1.1 COX-1 42
1.5.1.1.2 COX-2 43
1.5.1.2 COX derived mediators of inflammation and resolution 44
1.5.1.2.1 PGE_2 44
1.5.1.2.2 PGD_2 45
1.5.1.2.3 15deoxy$\Delta^{12-14}PGJ_2$ (15d-PGJ_2) 47
1.5.2 Adenosine 50
1.5.2.1 A2A receptor 51
1.6 Models of Chronic inflammation 53
1.6.1 Peritoneal Dialysis (PD) and encapsulating peritoneal sclerosis (EPS) 54
1.6.1.1 Peritonitis 54
1.6.1.2 Encapsulating peritoneal sclerosis 57
1.6.2 Chronic granulomatous disease 58
1.7 Objectives of this thesis 59
1.7.1 Hypothesis 59
Chapter 2 Materials and methods 61
2.1 Animal Husbandry 62
2.2 hPGD_2 synthase knockout mice (h$PGD_2S^{-/-}$) 62
2.2.1 Generation of H-PGD_2S gene-disrupted mice 62
2.2.2 Genotyping of mice 64

2.2.2.1 Tissue Digestion 64
2.2.2.2 Polymerase Chain reaction 64
2.3 Study of Peritonitis 65
2.3.1 Murine Peritonitis 65
2.3.1.1 Preparation of Zymosan Type A for experimentation 66
2.3.1.2 Preparation of Group B streptococcus (GBS) for experimental peritonitis\ 66
2.3.1.3 Induction of murine Peritonitis 66
2.3.1.4 Measurement of cell numbers 67
2.3.1.5 Trypsinization of peritoneal cavity 67
2.3.2 Human Peritonitis 67
2.4 Analysis of leukocyte profile and function 68
2.4.1 Flow cytometry 68
2.4.2 Leukocyte separation 71
2.4.2.1 Isolation of lymphocytes from peritoneal lavage 71
2.4.2.2 Negative isolation of T cells 71
2.4.2.3 Negative isolation of B cells 72
2.4.3 Adoptive transfer of lymphocytes into gp91^{phox} knockout mice 72
2.4.4 Macrophage isolation and culture 73
2.4.4.1 Isolation of macrophages from peripheral blood sample 73
2.4.4.2 Culture of bone marrow derived macrophages 74
2.4.4.2.1 L929 supernatant 74
2.4.4.3 Culture of peritoneal macrophages 75
2.4.5 Stimulation of leukocytes 75
2.4.6 Macrophage clearance assays 75
2.5 Measurement of apoptosis 77
2.5.1 Flow Cytometry 77

2.5.2 Caspase-3 77
2.5.2.1 Quantification of protein 78
2.6 Eicasonoid analysis 79
2.6.1 Extraction of prostaglandins and Measurement of PGD_2 79
2.6.2 Immunoassay for PGD_2 79
2.6.3 Measurement of 15d-PGJ_2 by LC-MS-MS 80
2.7 Measurement of chemokines and cytokines 80
2.8 Measurement of Purines 81
2.9 Statistical analysis 82
Chapter 3 The characterisation of inflammation in $hPGD_2S^{-/-}$ mice in response to zymosan induced peritonitis 83
3.1 Introduction 84
3.2 Materials and Methods 86
3.3 Results 87
3.3.1 Acute Inflammation 87
3.3.2 $hPGD_2S$-derived lipid mediators in acute inflammation 90
3.3.3 Cytokine and chemokine profile in $hPGD_2S^{-/-}$ and wild type mice 92
3.3.4 The role of PGD_2 receptors- DP1 and DP2 in zymosan peritonitis 94
3.3.5 The role of 15-Deoxy-Delta-12, 14-prostaglandin J_2 [15d-PGJ_2] in acute murine peritonitis in $hPGD_2S^{-/-}$ mice 96
3.3.6 Resolution of Inflammation 98
3.3.7 Lymphocytes profile during resolution phase 100
3.3.8 Failure of Macrophage efflux from the inflamed peritoneal cavity 101
3.3.9 Effect of $hPGD_2S$ on resident macrophages and lymphocytes ex vivo 104
3.3.10 Conclusions 107
3.4 Discussion 108

Chapter 4 Biphasic trafficking of lymphocytes during acute inflammation 112
4.1 Introduction 113
4.2 Material and Methods 114
4.2.1 Adoptive transfer of lymphocytes 115
4.3 Results 115
4.3.1 Lymphocyte profile in human peritonitis 115
4.3.2 Lymphocyte profile in murine zymosan peritonitis 116
4.3.2.1 T Lymphocytes 117
4.3.2.2 B Lymphocytes 117
4.3.3 Peritoneal lymphocyte disappear in response to PGD_2 120
4.3.4 Resolution phase lymphocytes are protective against super-infection 122
4.3.5 Deficiency of repopulating lymphocytes in non-resolving chronic granulomatous disease 125
4.3.6 Conclusions 127
4.4 Discussion 128
Chapter 5 The profile of adenosine and its metabolites in peritoneal inflammation of gp91phox$^{-/-}$ mice, a model of non resolving inflammation 134
5.1 Introduction 135
5.2 Material and Methods 137
5.3 Results 138
5.3.1 Exaggerated inflammatory response in gp91$^{phox-/-}$ mice 138
5.3.2 Reduced synthesis of purines and cAMP in gp$^{91phox-/-}$ mice 139
5.3.3 Adenosine and cAMP profiles in human resolving peritonitis 142
5.3.4 Adenosine, *via* A_{2A} is anti-inflammatory and not resolution-toxic 143
5.3.5 Anti inflammatory but not pro-resolution effect of adenosine *via* A_{2A} in gp91$^{phox-/-}$ mice 146

5.3.6 Conclusions 148
5.4 Discussion 149
Summary 154
Limitations of the study 158
Future work 159
References 161
Manuscripts of the publications based on this thesis 191

Index of Figures

Chapter 1

Figure 1.1 The cardinal signs of inflammation…… 1

Figure 1.2 The leukocyte profile during acute inflammation and resolution 22

Figure 1.3 Dendritic cell sub-types 34

Figure 1.4 The Prostaglandin pathway.....40

Figure 1.5 Lipoxigenase pathway 42

Figure 1.6 Adenosine metabolism.....51

Chapter 2

Figure 2.1 Structure of the wild-type H-PGDS allele, the targeting vector, and the targeted allele 63

Figure 2.2 Bands of WT and KO alleles visulaised by UV transilluminator.....65

Figure 2.3 FACS plots for different leukocyte sub-populations 69

Figure 2.4 GR-1 and F-80 profile in the first 24 hours following zymosan.....70

Figure 2.5 Dual labelling for PKH26-PCL and F4/80 76

Chapter 3

Figure 3.1 Zymosan induced peritonitis in wild type C57blackVI and $hPGD_2S$ deficient mice. .89

Figure 3.2 $hPGD_2S$ synthesises 15d-PGJ_2 during zymosan-induced resolving peritonitis. 91

Figure 3.3 Cytokine and chemokine levels in $hPGD_2S^{-/-}$ and wild type mice in zymosan peritonitis.....93

Figure 3.4 The role of DP1 and DP2 receptor agonists in acute zymosan peritonitis.....95

Figure 3.5 The role of 15d-PGJ_2 in zymosan peritonitis in C57blackVI wild type mice. 97

Figure 3.6 Lymphocyte profile of wild type and hPGD2S-/- mice following zymosan peritonitis by FACS.....99

Figure 3.7 Efflux of macrophages during resolution in hPGD$_2$S-/- and wild type mice following zymosan peritonitis.....103

Figure 3.8 The differential effect of hPGD$_2$S on the inflammatory phenotype of peritoneal leukocytes.....106

Chapter 4

Figure 4.1 Peritoneal cellular profile in patients undergoing peritoneal dialysis who develop acute resolving peritonitis 116

Figure 4.2 T lymphocyte profile in murine zymosan-induced peritonitis.....118

Figure 4.3 B lymphocyte profile in murine zymosan-induced peritonitis. 119

Figure 4.4 PGD$_2$ controls the clearance of peritoneal-resident lymphocytes.....121

Figure 4.5 Zymosan peritonitis in lymphocyte-deplete RAG2$^{-/-}$ mice.....124

Figure 4.6 The absence of repopulating lymphocytes during non-resolving inflammation 126

Figure 4.7 A summary of the scheme of events that occurs in acute inflammation with reference to lymphocyte trafficking 133

Chapter 5

Figure 5.1 Zymosan induced peritonitis in gp91$^{phox-/-}$ and wild type C57black VI mice 139

Figure 5.2 Arachidonic acid and adenosine metabolism in gp91$^{phox-/-}$ mice compared to wild types 141

Figure 5.3 Expression of adenosine and cAMP concomitant with resolution of human peritonitis. 142

Figure 5.4 Investigation of Adenosine, *via* A_{2A} being anti-inflammatory and not resolution-toxic. 145

Figure 5.5 Demonstration of Adenosine, *via* A_{2A} being anti-inflammatory but not pro-resolution in gp91$^{phox-/-}$ mice.....147

Index of Tables

Chapter 1

Table 1.1 Mediators of inflammation 19

Table 1.2 Effect of adenosine on T cell subsets 52

Table 1.3 Organisms responsible for peritoneal dialysis peritonitis 56

Chapter 2

Table 2.1 Antibodies used for FACS analysis of murine peritoneal leukocyte exudate 69

Chapter 3

Table 3.1 Lymphocyte subsets in $hPGD_2S^{-/-}$ and wild type mice. 100

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Declarations

Chapter 3

Dr Mark Hilliard at the Conway Institute, Dublin, Ireland, carried out measurement of 15d-PGJ_2 by LC-MS-MS.

In the experiments dealing with macrophage efflux from peritoneal cavity, the parathymic nodes were processed and examined for PKH26-PCL labelled macrophages in the laboratory of Dr Geoff Bellingan, University College, London.

Chapter 4

Professor Derek Gilroy carried out animal experiments with Rag2-/- mice at the biological services unit at the Kennedy Institute, London together with Dr Toby Lawrence.

Chapter 5

The measurements of cAMP, inosine and adenosine were carried out in the laboratory of Professor Edwin Jackson, Center for Clinical Pharmacology, Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Carrageenin induced pleurisy in rats which examined the role of A2A receptor agonist CGS21680 and PDE4 inhibitor rolipram (Figure 5.4A and 5.4B) were carried out by Priscilla Swamynathan at Experimental Pathology, William Harvey Research Institute, Charterhouse square, London.

Publications from this thesis

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Rajakariar R, Newson J, Jackson EK, Sawmynaden P, Smith A, Rahman F, Yaqoob MM, Gilroy DW (2009). Non-resolving inflammation in gp91phox $^{-/-}$ mice, a model of human chronic granulomatous disease, has lower adenosine and cyclic adenosine 5'-monophosphate.
J Immunol 182(5): 3262-9.

Abstract

Inflammation is fundamentally a beneficial response leading to removal of the offending factor, and resolution, an active regulated process that is essential to maintain tissue integrity and function. Previous work in our department showed a role for COX-2 derived PGD_2 and its cyclopentenone metabolite 15d-PGJ_2 during resolution in a pleurisy model. I investigated mice lacking $hPGD_2S$ and therefore absent PGD_2 and 15d-PGJ_2 by inducing peritonitis with zymosan. The resolution of peritonitis in $hPGD_2S$ mice was delayed compared to wild type C57black VI mice. PGD_2 via its action on the DP1 receptor controls the balance of pro- versus anti-inflammatory cytokines that regulate leukocyte influx as well as monocyte-derived macrophage efflux from the inflamed peritoneal cavity to draining lymph nodes leading to resolution. Previous data that laid doubt to the presence of 15d-PGJ_2 in *in vivo* models and questioned its role in resolution of innate inflammation. By measurement of peritoneal exudates with LC-MS-MS, there is definitive proof that 15d-PGJ_2 is synthesised during mammalian inflammatory responses. When the cellular profile was examined by flow cytometry, a biphasic response of lymphocytes was observed with the disappearance during acute inflammation with activation of the DP-1 receptor. The profile of lymphocytes that reappear during resolution differed from the naïve state and comprised of B1, NK, gamma/delta T, $CD4^+/CD25^+$ and B2 cells. The lymphocytes do not appear to have a role in resolution as lymphocyte deficient $RAG2^{-/-}$ and wild types resolve uniformly. However repopulating lymphocytes are critical for modulating responses to superinfection as observed by the exaggerated peritonitis/death of $RAG2^{-/-}$ mice when exposed to group B streptococcus following zymosan. In $gp91phox^{-/-}$ mice, an experimental model of CGD, where the peritonitis failed to resolve, the adaptive transfer of resolution phase lymphocytes into the peritoneum, was protective against superinfection. Furthermore in wild type mice when the anti-inflammatory adenosine and downstream cAMP were measured in peritonitis, there was a biphasic response. In $gp91phox^{-/-}$ mice, both adenosine and cAMP were significantly lower at onset and again at resolution. Adenosine, signalling

through its A_{2A} receptor and therefore elevating cAMP is not only anti-inflammatory, but also importantly, it does not impair pro-resolution pathways as antagonism of the A_{2A} receptor worsens acute inflammation and prolongs resolution.

In summary, resolution is an active regulated process that requires the input COX-2 derived PGD_2 as well as anti-inflammatory A_{2A} receptor activation. To further refine this model it was shown that repopulating lymphocytes are essential to prevent super-infection and persistence of inflammation.

Chapter 1 Introduction

1.1 Overview of Inflammation

1.1.1 The necessity of the inflammatory response

Inflammation is primarily a host response to an injurious stimulus such as pathogens and chemical irritants. The cardinal sign of inflammation heat, redness, swelling, pain and loss of function, was first described by Celsus more than 2000 years ago (Figure 1.1)(Gilroy, Lawrence et al. 2004). This is fundamentally a beneficial response leading to removal of the offending factor and the restoration of tissue structure and function. However, as much as an inflammatory response is needed to neutralise the invading pathogen, it is equally important bring about resolution of inflammation to maintain the integrity of the tissue that was involved.

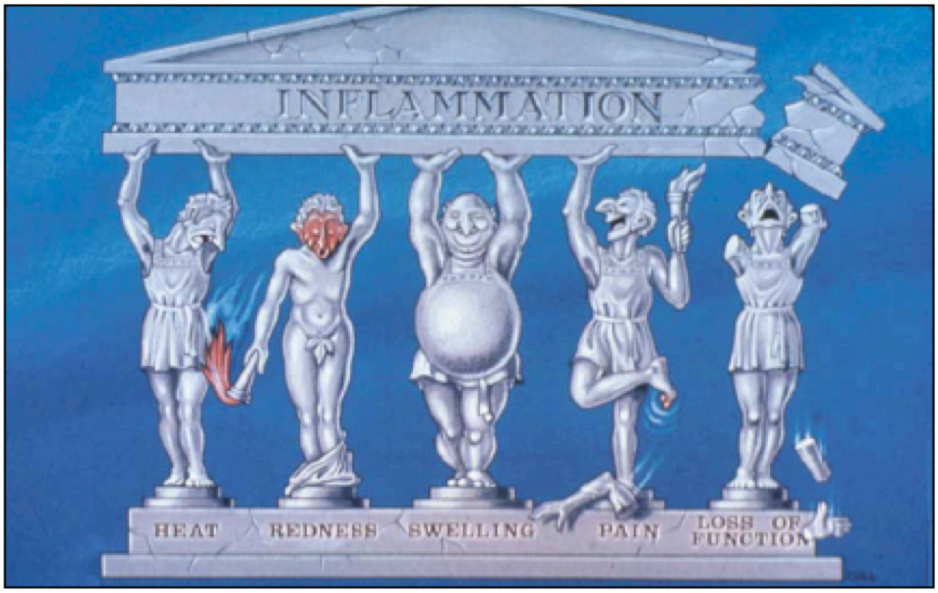


Figure 1.1 The cardinal signs of inflammation depicted by the 'The five Greeks'. Commissioned by the late Professor Derek Willoughby and drawn by P. Cull

This is mediated, in the first instance, by a host of signals that drive the inflammatory response in a manner well understood, which all require the up-regulation of cell adhesion molecules and expression of chemokines resulting in the accumulation of inflammatory leukocytes at the site of tissue injury. Once the inflammatory stimulus has been neutralised then inflammation can abate

(Nathan 2002; Serhan and Savill 2005). The whole purpose of inflammation is to remove the offending agent and subsequent restoration of tissue function. For example in lobar streptococcal pneumonia with antibiotic therapy the morphological changes caused by the infiltration of neutrophils and other inflammatory cells invariably resolves without any chronic tissue damage. This however does not happen in a passive manner as originally thought, where the inflammatory response was simply believed to "fizzle out". Quite the opposite, in-as-much as onset is highly controlled, resolution is also an active event managed by an increasing number of soluble mediators and mechanisms that culminate in inflammation switching off and importantly resulting in the stromal tissue that hosted the response returning to is prior physiological state.

1.1.2 The transition from acute inflammation to resolution

Acute inflammation is initiated by resident cells within the affected tissue (resident macrophages, dendritic cells, lymphocytes) and is characterised by changes in the microcirculation with resultant vasodilatation leading to movement of leukocytes from the blood into the microenvironment termed the extracellular matrix [ECM]. This is mediated by up-regulation of adhesion factors on the endothelial surface, which facilitate the adherence of inflammatory leukocytes and migration into the affected area (Lawrence, Willoughby et al. 2002). Table 1.1 summarises the spectrum of agents that drive the acute inflammatory process necessary to neutralise the offending agent. The hallmark of the acute phase is the influx of neutrophils within the first 24 hours in response to triggers for tissue injury such as invading microbes, surgical trauma that activate the release of pro-inflammatory cytokines, for example IL-6, IL-1β and TNFα. Arachidonate-derived prostaglandins such as PGE_2 from the cyclooxygenase pathway and 5-lipoxygenase pathway products leukotriene B_4 (LTB_4) amplify the movement of PMNs to site of injury(Gilroy, Lawrence et al. 2004). Once PMNs reach to site of injury, they remove the invading pathogen by the process of phagocytosis and neutralise by

the initiating an extensive intracellular armamentarium of degradative enzymes and reactive oxygen species (ROSs).

Mediator Class	Pro-inflammatory	Anti-inflammatory
Amines	Histamine, bradykinin	Adrenaline, noradrenaline
Lipid Mediators	PGE_2, LTB_4, LTC_4	PGJ_2, PGA_2, Lipoxins
Complement	C3a, C5a	C1q receptor
Cyclic nucleotides	cGMP	cAMP
Adhesion molecules	E-selectin, P-selectin, ICAM1, VCAM1	$\alpha 2\beta 3$ integrin, TSP receptor, PS receptor
Cytokines	TNF-α, IL-1β, IL-6	TGF-β1, IL-10
Chemokines	IL-8, MIP-1α, MCP-1	
Steroid hormones		Glucocorticoids

**Table 1.1 Mediators of inflammation PG Prostaglandin, cGMP- cyclic guanosine 3.6 monophosphate, cAMP-cyclic adenosine 3,5 monophosphate, I/VCAM-1- intracellular/Vascular adhesion molecule-1, TNF- Tumour necrosis factor, IL- interleukin, TGF-Transforming growth factor, MIP- Macrophage inflammatory protein, MCP-1 Monocyte chemotactic protein (*adapted from (Lawrence, Willoughby et al. 2002)*

However, continued influx and persistent neutrophil activity will mean that the acute inflammation remains unchecked leading to oedema and permanent tissue damage. Conventional wisdom was that once the source for acute inflammation (invading pathogen, chemical injury) has been removed then the stimulus to activate pro-inflammatory mediators and cytokines is lost leading to reduced efflux of leukocytes allowing for the tissue to restore structure and function. It is now appreciated that just as the acute inflammatory process is an active response to injury, resolution does not occur in a passive manner and is mediated by a sequential release of cytokines and lipid mediators summarised in table 1.1 that is discussed in detail later in this

chapter. The resolution involves reversing vascular permeability, the switching off of leukocyte migration, enabling the disposition of leukocytes and debris away from the tissue and most importantly to limit excessive tissue injury and prevention of the development of chronic inflammation. *Timing here is the essence*. Just as delayed triggering of resolution lead to chronic inflammation, premature onset of signals of resolution will mean the failure of arresting the offending agent leading to overwhelming inflammation (e.g. uncontrolled septicaemia) that can lead to permanent disability or death of the host (Hotchkiss and Nicholson 2006). The signalling pathways that lead to the 'switch' from pro-inflammatory to pro-resolution mediator release begin early in inflammation and often involve the share the same pathways with differing end products (Serhan and Savill 2005). The 'class switch' including COX-2 metabolite PGE_2 to PGD_2 (and its metabolite 15-PGJ_2), leukotrienes (e.g. LTB_4) to lipoxins (e.g. LXA_4) and the generation of lipid mediators from omega-3 polyunsaturated fatty acids- resolvins and protectins, is mediated by the inflammatory leukocytes at different time periods during inflammation (Serhan and Savill 2005).

Therefore once the offending agent is neutralised, PMNs undergo apoptosis (Savill and Haslett 1995), which in turn is phagocytosed by tissue macrophages (Metchnikoff 1893; Savill, Dransfield et al. 1990), which increase in number during the resolution phase of the inflammation. Apoptosis of PMNs is preferred to necrosis, the latter that may promote further tissue injury. The onset of resolution is rapid, with PMNs falling by 50% within an interval of 8 hours following peak inflammation as evidenced in a resolving zymosan induced peritonitis model (Bannenberg, Chiang et al. 2005). The phenotypes of the macrophage population that assist in resolution (termed resolution phase macrophages, Rm or M2) differ in phenotype to that of the resident or 'inflammatory' population (M1) as in Rm cells express TGFβ1 which suppresses pro-inflammatory signalling from toll like receptors, the latter a critical mediator of innate immunity (Zhang, McCluskey et al. 1998; Gordon 2003; Verreck, de Boer et al. 2004).

Finally along with the removal of the PMNs, macrophage numbers fall as the inflammatory cells are cleared via the draining lymphatics with restoration of normal tissue structure and function(Bellingan, Caldwell et al. 1996). In this milieu there also observed the disappearance of lymphocytes in acute inflammation and re-population during resolution whose role (if any) is poorly understood. Figure 1.2 illustrates the cellular profile in both acute and resolution of inflammation. The principle aim as mentioned above is restoration of tissue structure and function. However, if the tissue injury is widespread as in a large wound infection, the tissue repairs itself by inward growth of surrounding tissue with vascular supply from proliferating capillary loops with movement of leukocytes and fibroblasts. This is termed granulation tissue which eventually leaves a fibrous scar (Gurtner, Werner et al. 2008) and should not be mistaken as failure of resolution as the resultant fibrous scar is a reflection of the insult being greater than the capacity of the host to restore complete function.

As much as the changes in cellular profile in acute inflammation is important to restore tissue architecture, enhancing the ability of vertebrates to fight infection that they have previously encountered has been important for its evolution and survival. Therefore the antigen presenting cells, dendritic cells, in the site of inflammation also remove the offending agent by phagocytosis and then present antigenic peptides complexed with major histocompatibility molecules (MHC) in the lymphoid organs critical for the development immunological memory, hallmark of adaptive immunity.

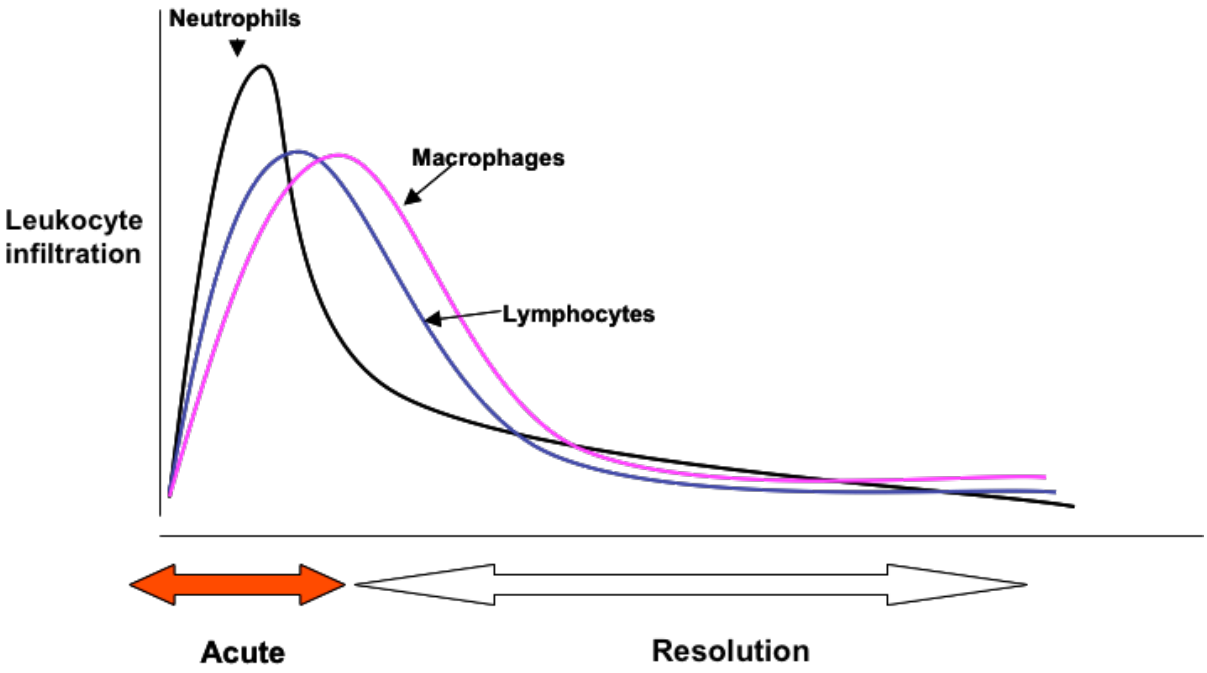


Figure 1.2 The leukocyte profile during acute inflammation and resolution

1.1.3 Wound Healing

It's important to distinguish the traditional concept of acute inflammatory resolution in response to an injurious agent to that of wound healing where the organ repairs itself following injury to the outer protective barrier (Gurtner, Werner et al. 2008; Sonnemann and Bement 2011). The process can be divided into three overlapping stages: inflammation, new tissue formation and remodelling. The epidermis and dermis of the skin act as a protective barrier and when breached, activated platelets form a blood clot that acts as a scaffold for infiltrating cells first being PMNs that release more cytokines. PMN influx is followed by macrophage infiltration that further exacerbates the phagocytic activity important to scavenge foreign objects, organisms and apoptotic PMNs. As inflammation progresses to neutralise the offending event, the process of new tissue formation with re-epithelialisation begins with migrating activated fibroblasts together with macrophages forming the granulation tissue which replaces the fibrin clot in the healing wound. Angiogenesis aids this process by increasing blood flow to deliver nutrients and oxygen in order to facilitate healing. This process gradually evolves into remodelling with

involution of the granulation tissue together with dermal regeneration. This involves apoptosis of myofibroblasts, endothelial cells and macrophages. The aim of successful wound healing process is to restore tissue architecture to its pre-inflamed, physiological state.

1.2 Apoptosis and Phagocytosis

1.2.1 Apoptosis

As important as it is for PMNs to neutralise the offending agent, if they were to remain and function unchecked, it will lead to persistent inflammation and damage. Therefore for resolution to occur the inflammatory leukocytes needs to be cleared from the site of injury, one method being apoptosis (Savill and Haslett 1995). This is a process, by which redundant, damaged or infected cells are eliminated from tissues and therefore being protected from the alternative that is necrosis when noxious substances from the dying cells are leaked into the tissue.

Morphologically this is characterised by cell shrinkage, cytoplasmic condensation, nuclear and chromatin fragmentation leading to the formation of an apoptotic body, which is then, engulfed by a phagocyte, namely monocyte derived macrophages. In innate inflammation, this is the principle mechanism by which inflammatory leukocytes, namely PMNs are removed from site of its action.

The execution switch for apoptosis is a group of previously dormant proteases, the caspases that is activated by two distinct pathways. The *extrinsic*, that engage the so-called surface 'death receptors'- TRAIL (TNF related apoptosis inducing ligand); TNFα, CD95L, initiate caspase-8 mediated pathways. The second, *intrinsic* mitochondrial initiated caspase-9 mediated pathway is provoked by cell stress due to inadequate cytokine support for the cell or diffused intracellular damage, which is regulated by the Bcl-2 family (Roy and Nicholson 2000; Danial and

Korsmeyer 2004; Gupta 2005). Both caspase-8 and-9 can activate caspase-3, the critical final common pathway to induce apoptosis.

Following the programmed death of the leukocyte during inflammation, the apoptotic body must be cleared from site of injury by phagocytosis by scavenging monocyte derived macrophages that employ two classes of adhesion molecules, integrin heterodimers of the β_3 family and thrombospondin -1 (TSP-1) receptor (Savill, Dransfield et al. 1990; Savill, Hogg et al. 1992). Therefore apoptotic PMNs bind to macrophages via $\alpha_v\beta_3$ vitronectin receptor integrin and TSP-1 receptor CD36 leading to the phagocytosis. Many other macrophage surface molecules have also been implicated in clearance of apoptotic leukocytes including lectin, phosphatidylserine receptor 61D3 and class A scavenger receptors (Savill 1997).

1.2.2 Phagocytosis

Phagocytosis is the process by which invading pathogens are taken and neutralised by the inflammatory cell such as PMNs, dendritic cells and macrophages. This is important for both the innate immune response as well as for antigen presentation and the development of adaptive immunity. Following uptake of the pathogen, it is trapped together with extracellular fluid in a vacuole termed the phagosome (Flannagan, Cosio et al. 2009). This undergoes a series of changes with sequential interaction with endosomes during which the vacuole lumen becomes increasingly acidic (Desjardins, Huber et al. 1994). Apart from the decrease in the pH, the lumen is enriched with degradative proteases and lysosomal associated membrane proteins (LAMP). This culminates with fusion with the lysosome to form the phagolysosome, which is highly acidic (pH 4.5) and contain an extensive repertoire to neutralise invading pathogens. The microbicidal activity within the phagolysosome is mediated by the acidity that inhibits pathogen growth and activity, degradative proteases and peptides (e.g. Latoferrin, cathelicidin, lyzozymes, cysteine proteases) and reactive oxygen species (ROS) (Flannagan, Cosio et al. 2009). For

instance during activation of neutrophils the heterodimer, gp91^{phox} and p22^{phox} (also known collectively as the cytochrome *b*558), components of the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase (NOX) migrate to the surface of phagosome or cellular membrane. This leads to the production of reactive oxygen species (ROS), superoxide with a direct microbicidal effect as well as the arrest of pathogen survival and growth (Quinn and Gauss 2004). Generation of ROS leads to the consumption of oxygen termed 'respiratory burst'. The importance of ROSs in elimination of microbes is highlighted by chronic granulomatous disease (CGD) where mutation in gp91phox subunit of NADPH oxidase, is characterized by recurrent infections that can result in death (Heyworth, Cross et al. 2003). The NOXs and CGD will be described in detail later in this chapter (*1.6.2 Chronic Granulomatous Disease*).

1.3 Innate versus Adaptive Immunity

Immunity, the state of protection from infectious disease, can broadly be divided into two arms: innate, and more specific adaptive immunity. The innate immune response is the first line of defence to invading pathogens and is independent of prior exposure to the pathogen (Turvey and Broide 2010). Adaptive immunity develops when the body is exposed to various antigens expressed by pathogens and builds a defence that is specific to those antigens which typically develops within five to six days of exposure. Innate and adaptive immunity co-operate to generate a *'total'* response more efficient than either alone (Turvey and Broide 2010).

1.3.1 Innate Immunity

Innate immunity plays a critical role in host defence against infection. It confers its protective effect by anatomic [physical barriers], physiologic [inhibition of growth and survival of pathogens] and by the inflammatory response as described previously. This process has remained conserved through evolution and is the principle mechanism by which most organisms respond

to infection and cellular injury. Only vertebrates have an additional adaptive immune system. The recognition of pathogens is through highly conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, mannans and bacterial CpG DNA motifs, common to a large group of microorganisms but absent in the host as well as damage-associated molecular patterns (DAMPs), endogenous products released from damaged and dying cells (Seong and Matzinger 2004). They are recognised by pattern recognition or germ line-encoded recognition receptors. There are two families of receptors that recognise the above mentioned integral structural components of microbes, Toll-like receptor (TLR) family (Takeda, Kaisho et al. 2003; Akira and Takeda 2004) and proteins containing nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains (Chamaillard, Girardin et al. 2003).

Toll-like receptors, comprise of conserved leucine-rich repeat (LRR) ectodomain, transmembrane and cytoplasmic tail domains. The latter contains the canonical Toll/interleukin 1 (IL-1) receptor domain and is involved in downstream signalling process. LRR domain is important in ligand binding and its high specificity has led to identification of 10 and 12 subtypes of TLRs in humans and mice respectively. TLR 1-9 is conserved in both species. TLRs are expressed on macrophages, dendritic cells, B cells, specific types of T cells and also on non-immune cells such as fibroblasts and epithelial cells. The recognition of PAMPs by TLRs occurs in various cellular compartments including the plasma membrane, endosomes, lysosomes and endolysosomes. Therefore TLRs 1,2,4,5 and 6 are expressed on the cell surface whereas TLR 3, 7-9 are exclusively intra-cellular. The localisation and accessibility to ligand is tightly regulated in order to maintain tolerance of self-antigen and downstream signal transduction (Takeda, Kaisho et al. 2003).

However as previously mentioned, as much as inflammation is a necessary to counteract the injurious effects of the foreign organism, there has to be down regulation of TLR signalling to

limit the impact of pro-inflammatory signalling pathways. Therefore there are pathways such as one that involves Triad3A, a ubiquitin ligase which binds to TLR-4 and TLR-9 leading to its degradation and reduced TLR 4- and TLR 9 mediated NF-κB activation, one of the numerous natural 'stop' signals to prevent an exaggerated response early in the inflammatory cascade (Chuang and Ulevitch 2004).

1.3.2 Adaptive immunity

During vertebrate evolution, the ability to develop antigen specific immune response together with development of immunological memory became essential to deal with pathogens that were able to mutate to avoid host detection and evade the innate immune response. In contrast to innate immunity, which is geared for rapid sensing and elimination of pathogens, the adaptive immune response requires antigen presentation. The cells of the adaptive immune system include antigen-presenting cells i.e. dendritic cells, T lymphocytes that mature in the thymus and immunoglobulin secreting B-lymphocytes, which arise in the bone marrow (Bonilla and Oettgen 2010). These facilitate antigen specific pathways, immunological memory and the ability to recognise self from non-self antigens.

Following infection, pattern-recognition receptors expressed by antigen-presenting cells (APCs) such as the mannose receptor bind to PAMPs such as LPS, which mediate the uptake and delivery of pathogen to lysosomes. Professional APCs such as migratory dendritic cells are ubiquitous in regions most vulnerable to foreign insult such as the skin and mucosal surfaces. The pathogen is then degraded as part of the phagocytic process, and the foreign antigenic peptides are presented on the surface of the APCs complexed with major histocompatibility (MHC) molecules. These cells then home into to the lymph nodes where lymphocytes are activated.

$CD4^+$ T helper (T_h) cells activation is initiated when the T cell receptor (TCR) recognise the antigen peptide/MHC complex on an APC. This leads to the formation of the 'immunologic synapse' that comprise of CD3, TCR, which bind to the peptide/MHC class 2 complex, as well as the CD4 molecule. This interaction is further stabilised by integrins. Effector T cells then secrete various cytokines which in turn activate B cells, cytotoxic (Tc) cells, macrophages and various other cells that participate in the immune response.

In order to achieve optimal lymphocyte activation a second signal is necessary to complement the antigen-MHC-TCR interaction in T cells and immunoglobulin- receptor in B cells and this is termed co-stimulation (Lafferty, Andrus et al. 1980; Brunet, Denizot et al. 1987; June, Ledbetter et al. 1990). For this to occur a vast array of co-stimulatory molecules exist on the surface of naïve T and B-lymphocytes that bind to its ligands on APCs and T helper cells respectively. During T cell activation, lack of co-stimulation leads to failure to sustain proliferation, the inability to mount a cytokine response, increased lymphocyte apoptosis and development of anergy i.e. unresponsive to antigenic stimuli (Schwartz 2003). The first known co-stimulatory molecule was CD28 on T cells that bind to B7.1 and B7.2 ligands on the APCs (Brunet, Denizot et al. 1987). Activated CD28 in turn up regulate CD40 ligand, which by binding to CD40 on APCs will in turn increase B7 expression. Furthermore, CD28 also induces T cell expression of ICOS (inducible co-stimulator molecule) that binds to its namesake ligand (Sharpe 2009). However co-stimulation needs to be kept in check to avoid uncontrolled T cell activation. The best studied is CTLA-4, a member of the CD28 family, whose expression is also increased with T cell activation which then inhibits T cell proliferation and IL-2 synthesis and therefore, co-stimulation is a self-limiting process.

1.4 Cellular components in inflammation and resolution

1.4.1 Granulocytes

This group of cells include Polymorphonucleor Neutrophils (PMN), eosinophils/mast cells and basophils. The hallmark of these cells is granules that contain proteins that kill microbes and digest tissues. Even though the PMN is the principle cell in the acute inflammatory response, the important role of mast cells in both innate and adaptive immunity must be appreciated.

1.4.1.1 Mast cells

In evolutionary terms mast cells have been conserved and humans' deficient of this group of inflammatory cells have never been reported (Weller, Collington et al. 2011). Even though mast cells have been implicated in the chronic detrimental effects due to asthma, allergy and anaphylaxis, they have an important role in immune homeostasis. Mast cells are strategically placed at the host-environment interface with the potential to be the first responders typically within seconds of pathogen invasion by releasing proteases [e.g.: MCP-1), IL-6, TNF following activation of TLRs by PAMPs leading to recruitment of PMNs and macrophages (Abraham and St John 2010; Weller, Collington et al. 2011). They are therefore able to communicate the presence of pathogens with cells in both innate and adaptive arms of inflammation- PMNs, macrophages, dendritic cells, B & T lymphocytes as well as endothelial, smooth muscle and epithelial cells.

1.4.1.2 Polymorphonuclear neutrophils

PMNs, which are generated in the bone marrow and circulate in the blood stream, are indispensable for the elimination of the invading pathogen. The production in the bone marrow is in a steady state and is increased in response to granulocyte colony stimulating factor (G-CSF) that occur when there is tissue injury or if microorganisms successfully overcome the physical

barriers (Lieschke, Grail et al. 1994). Hallmark of PMNs and other granulocytes are the presence of intracellular granules necessary for neutralising the pathogen. Neutrophil granules are classified into three distinct subsets based on the presence of characteristic granule proteins: primary (azurophil) granules (myeloperoxidase [MPO]), secondary (specific) granules (lactoferrin), and tertiary gelatinase granules (gelatinase) (Borregaard and Cowland 1997).

Following injury or invading microorganisms, endothelial cells are activated by cytokines such as TNFα and IL1β that lead to capture of PMNs by selectins. This leads to a process of rolling, and then adhesion to the endothelium followed by trans-endothelial migration mediated by Mac-1 and LFA-1. The neutrophils then phagocytose the invading pathogen, degranulate and then undergo apoptosis, with the apoptotic PMNs being scavenged by tissue macrophages (Borregaard 2010).

Following neutralisation of the offending agent, the 'spent' PMN either undergoes necrosis or apoptosis, the latter being preferred. ChemR23 expressed on macrophages, dendritic cells and activated endothelial cells, mediates via phoshpinositide-3-kinase (PI3), protein kinase B (Akt) signalling the phagocytic capacity of macrophages for uptake of apoptotic neutrophils and reduce risk of necrotic PMN cell death. It also down-regulates G-CSF to limit PMN activation locally, a necessary 'stop' signal to limit acute inflammation (Kaur, Adya et al. 2010; Ohira, Arita et al. 2010).

PMNs also neutralise the offending agent by the generation of neutrophil extracellular traps (NETs), a web of chromatin and serine proteases that trap and kill microbial organisms extending the antimicrobial activity beyond the lifespan of the neutrophil. This is distinct from apoptosis. The mechanism of NET formation is unclear (Brinkmann, Reichard et al. 2004). It is interesting to note that PMNs from newborns are partially deficient of NET production and demonstrate decreased microbicidal effect. Hydrogen peroxide generated by the NADPH

oxidase is necessary for NET formation latter being absent in PMNs of chronic granulomatous disease (CGD), a disease state of chronic inflammation due to absent NOX activity (Bianchi, Hakkim et al. 2009; Metzler, Fuchs et al. 2011).

1.4.2 Mononuclear cells

1.4.2.1 Monocytes and macrophages

Monocytes, macrophages and dendritic cells originate from haematopoietic stem cell progenitors in the bone marrow. Under normal circumstances monocytes circulate for a short time before undergoing spontaneous apoptosis (Auffray, Sieweke et al. 2009). Monocytes may however escape their apoptotic fate by differentiating into macrophages with a longer life span (months to years) triggered by differentiating factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and colony-stimulating factor-1 (CSF-1). They are defined by the presence of the CSF-1 receptor (M-CSF, CD115). Monocytes differ from circulating dendritic cells (cDC) by the absence of MHC class 2 antigens or the integrin CD11c. Monocytes may also differentiate into inflammatory dendritic cells, the latter an essential component of the adaptive immune response. Activation of monocytes/macrophages occurs through the stimulation of TLRs leading to production of pro-inflammatory IL-1β and TNFα. The increased production of monocyte chemoattractant protein-1 (MCP-1/CCL2) contributes further to increase production of pro-inflammatory cytokines (Auffray, Sieweke et al. 2009).

In human there are three subsets of monocytes based on expression of CD14 and CD16. They are $CD14^{+}CD16^{-}$, which comprise 80-90% of circulating monocytes, $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{+}$ (Grage-Griebenow, Flad et al. 2000; Ziegler-Heitbrock 2000). In mice, monocytes are identified based on the side scatter on flow cytometry, CD115, dectin-1, CD11b

and expression of Gr1/Ly6C antigen. Ly6C is also expressed in granulocytes, NK cells (40%) and subsets of dendritic cells. Based on the expression of Gr1 and Ly6C, two subsets of monocytes have been identified in mice, the 'inflammatory' GR-1^{+}Ly6c^{high} (the phenotypic equivalent of CD14^{+}CD16^{-} in humans) and 'patrolling' GR-1^{-}Ly6c^{low} monocytes (Auffray, Sieweke et al. 2009). Following infection, increased numbers of the inflammatory monocytes leave the bone marrow that express TNFα and IL-1. They are selectively recruited to inflamed tissue and lymph nodes where they transform to M1 type macrophages and inflammatory dendritic cells both that perform effector functions during acute inflammation. The patrolling subtype, GR-1^{-}Ly6c^{low} monocytes are seen on intravital microscopy to be crawling on the luminal surface of vascular endothelium and survey the surrounding tissues for damage and infection. Furthermore, GR-1^{-}Ly6c^{low} monocytes initiate differentiation of the 'alternative' or M2 macrophage phenotype involved in inflammatory resolution and tissue healing (see below) (Martinez, Gordon et al. 2006; Auffray, Sieweke et al. 2009).

Macrophages are characterised by the surface expression of CD14, CD16, CD11b, and CD68 and additionally in mice, F4/80 (Taylor, Martinez-Pomares et al. 2005). Two types of macrophages have been characterised, M1 and M2. M1 cells assist Th1 immune responses mediated by IFN-γ, IL-1β and TNFα (with low levels of anti-inflammatory IL-10) and are involved in antigen presentation to B cells. Furthermore these cells also demonstrate increased expression of COX-2, iNOS derived NO, peroxynitrite, peroxide and superoxide (Zhang, McCluskey et al. 1998; Mantovani, Sica et al. 2004; Verreck, de Boer et al. 2004). To counterbalance the effects of the pro-inflammatory M1 cells, tissue protective Th2 by cytokines IL-4, IL-10 and TGFβ1 production characterise an alternative macrophage population, the M2 phenotype (Gordon 2003). There is, however, heterogeneity in this population based on the surface marker expression. For instance, in a resolving peritonitis model the resolution phase macrophages share expression of COX-2 and iNOS typical of M1 cells (Bystrom, Evans et al.

2008). The switch between macrophages typical of M1 versus the pro-resolution M2 is influenced by cAMP with the inhibition of the latter favouring the pro-inflammatory phenotype.

As inflammation resolves, monocytes/macrophages numbers in sites of inflammation fall. The disappearance is either by apoptosis, which is caspase-3 dependent (Parihar, Eubank et al. 2010), or via draining lymphatics (Bellingan, Caldwell et al. 1996; Bellingan, Xu et al. 2002). More recent work done in a peritoneal model of inflammation [sterile zymosan induced peritonitis], macrophages with the M1 phenotype is thought to be pro-inflammatory and aiding the acute inflammatory response, whereas the M2 macrophages is actively involved in promoting resolution (Bystrom, Evans et al. 2008).

1.4.2.2 Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells critical for adaptive immunity. Monocytes, macrophages and dendritic cells share a common macrophage-dendritic cell progenitor (MDP) (Fogg, Sibon et al. 2006) but there is a DC progenitor (CDP) that exclusively produces dendritic cells (Liu, Victora et al. 2009).

Dendritic cells can further be classified as conventional DCs and non-conventional DCs. The latter is not seen in a steady stage and develops in the presence of inflammatory stimuli. Conventional DCs are further sub-classified to migratory, which are able to carry antigen-MHC class 2 complexes from periphery (e.g. skin, lung) and migrate to draining lymph nodes where interaction with lymphocytes occurs. In contrast, lymphoid DCs are resident in lymphoid tissue and lack the migratory capacity. Non-conventional DCs differ by their capacity to express δIFN and can further be sub-classified into plasmacytoid and monocyte derived DCs, further illustrated by Figure 1.3 (Kushwah and Hu 2011).

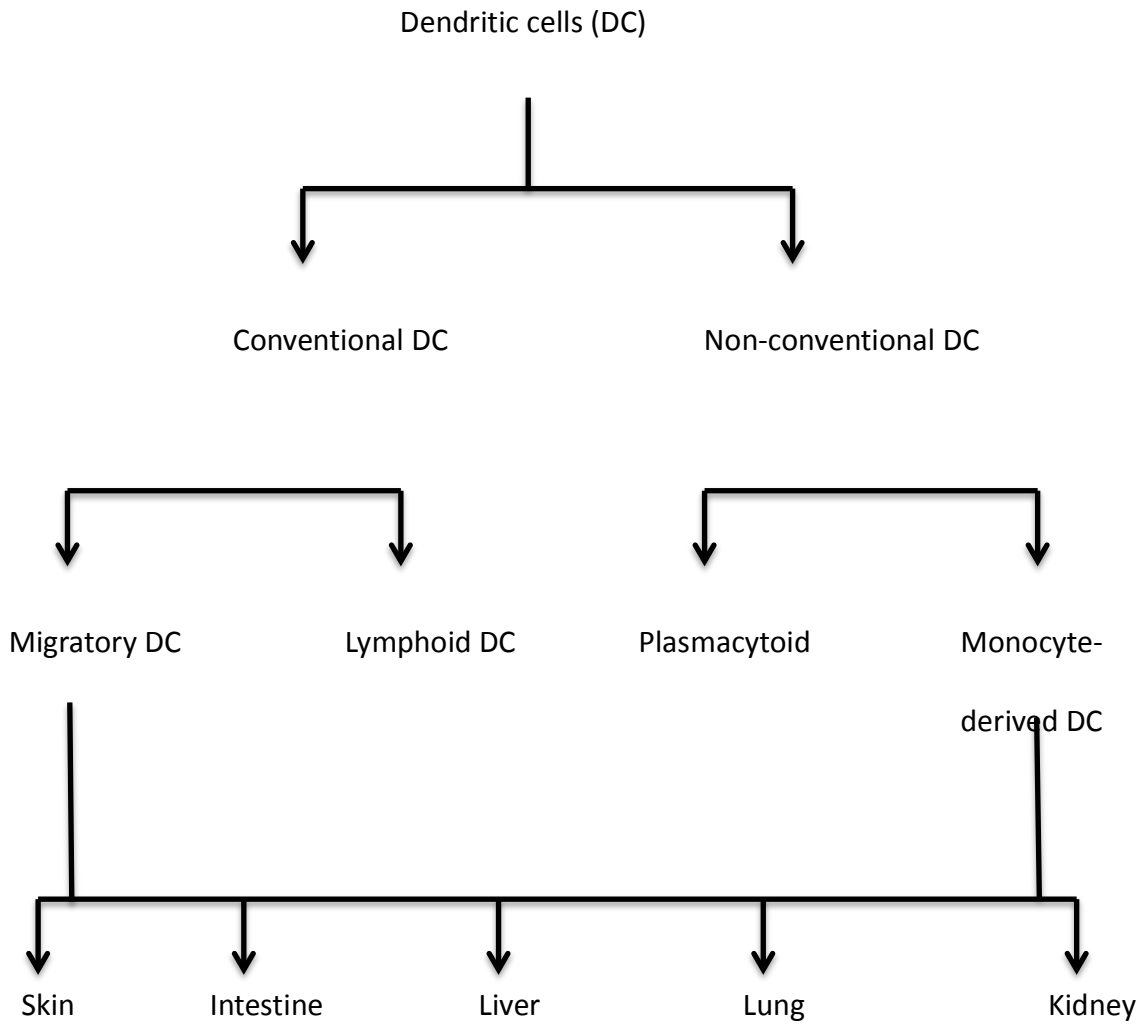


Figure 1.3 Dendritic cell sub-types

1.4.2.3 Lymphocytes

Predominant subsets of lymphocytes are T and B cells. Natural Killer cells (NK cells) are classified separately as a lymphocyte subclass and play a role in the innate immune system that kill cells by release of small cytoplasmic granules called granzyme and perforin (Cooper, Fehniger et al. 2001; Lanier 2005). At an evolutionary level they are more primitive and play an important role in defence against pathogens, virally infected cells and tumour cells. In humans they express CD16 and CD56 and in a majority CD8.

1.4.2.3.1 T cells

T cells that undergo maturation in the thymus are distinguished from B cells and NK cells by the expression of the $CD3^+$ T cell receptor (TCR) (Takahama 2006; Hedrick 2008). They play a central role in cell-mediated immunity and are responsible for immunological memory.

T helper cells (T_h), characterised by the expression of CD4 on its surface, is essential for maturation of B cells into immunoglobulin producing plasma cells and the activation of cytotoxic T cells. Therefore their effect against foreign organisms or autoimmunity is indirect. T_h cells can further be subdivided based on the cytokine profile (Table 1.2). T_h1 cells produce IFN-γ whereas T_h2 cells produce IL-4, IL-5, IL-6 and IL-13. T_h1 cell production is IFN-γ dependent, inhibited by IL-4 whereas the converse is true for T_h2 cells (Bonilla and Oettgen 2010). In response to an antigen, critical determinant to activation of the T_h cell is the cytokine profile by which naïve T helper cell is activated. IL-12 interacts with naive T_h lymphocytes to activate Signal Transducer and Activator of Transcription 4 (STAT4) leading to expression of the transcription factor T-box expressed in T cells (T-bet) that is responsible for differentiation to T_h1 phenotype, the essential component of cell mediated immunity. It simultaneously blocks the expression of Th2 cytokines and negatively regulates T_h17 differentiation. IL-4 is the principle determinant of Th_2 differentiation. IL-4 activates STAT6 that in turn promotes expression of GATA-3 and suppresses expression of T-bet. Th2 cells are essential for host humoral immunity to various infections including extracellular parasites such as helminths and are responsible for the development of allergic inflammatory disease such as asthma. T_h 17 cells are a more recently discovered subset that produces IL-17, thought to play a role in autoimmunity (Fouser, Wright et al. 2008).

Family	Cytokine repertoire
T_h1	IFN-γ, TNF-α, IL-2, IL-3
T_h2	IL-2, IL-4, IL9, IL-13
T_h17	IL-17, IL-21
Treg (CD25+Foxp3+)	IL-10

Table 1.2 Subtypes of CD4+ T_h cells

In addition to subsets of T_h cells, several families of CD4 positive regulatory T cells [T-reg] have been identified that include IL-10 producing peripherally differentiated (induced) Treg (iTreg) cells, thymic derived $CD25^+$ natural T reg cells. Thymic derived T reg cells are characterised by the constitutive expression of the transcription factor Forkhead box P3 (FOXP3) (Shevach, DiPaolo et al. 2006; Vignali, Collison et al. 2008).

1.4.2.3.2 B-lymphocytes

B-lymphocytes are generated by haematopoietic stem cell progenitors. The classic role of B lymphocytes is in the adaptive immune response with antigen presentation, production of high affinity protective immunoglobulins, up-regulation of co-stimulatory molecules, production of cytokines and regulation of T cell repertoire and response (Roosnek and Lanzavecchia 1991; Yi, Klinman et al. 1996; Lee and Koretzky 1998). This is mediated by the predominant B-2 cell subset, accounting for 85% of splenic and over 99% of circulating B cells (von Boehmer and Melchers 2010). However with the discovery of expression of TLRs on murine B cells, another subset (named B-1), characterised by the surface expression of $CD5^+$ was discovered with an important role in innate immunity (Hayakawa, Hardy et al. 1983; Wortis and Berland 2001; Dorshkind and Montecino-Rodriguez 2007). B-1 cells are relatively sparse in the spleen (2%)

but are the predominant resident B cell subset in peritoneal and pleural cavities occur earlier than B-2 during lymphocyte development (Godin, Garcia-Porrero et al. 1993). B-1 cells traffic continuously through the omentum that is mediated by peritoneal macrophage derived CXCL13 (Ansel, Harris et al. 2002).

With the discovery of the innate arm of lymphocyte biology, it is widely appreciated that B cells, in particular B-1 act as a bridge between innate and adaptive immune responses (Viau and Zouali 2005). The resident B-1 cells in pleural and peritoneal cavities are not continuously replenished unlike B-2 cells, which have a half-life of 5 months (Hao and Rajewsky 2001). T lymphocytes and B-2 cells that emerge are 'negatively selected' in order to remove self-reactive cell population in order to prevent auto-immunity but B-1 are 'positively selected' that produce self-reactive antibodies which also cross react with many pathogens and important for innate immunity.

B-1 cells, by producing a steady state of IgM, are able to neutralise invading pathogens early during infection by inhibiting replication and through complement binding (Baumgarth 2011). They are activated by TLR agonist such as lipopolysaccharide [LPS] and are unresponsive to B cell receptor activation as opposed to B-2 cells (Ansel, Harris et al. 2002; Baumgarth 2011). Following activation, B-1 cells migrate from the peritoneal cavity to the spleen or mucosal tissues where they differentiate to IgM or IgA secreting cell. Furthermore in the gut mucosa and respiratory tract, it acts as the first line of defence by polyspecific production of IgA. In tissue homeostasis, IgM bind and neutralise altered self antigens on apoptotic cells preventing autoantibody production, decrease development of atherosclerosis by binding to lipoproteins and enhance phagocytosis by dendritic cells. In IgM deficient mice B-1 cells are increased by almost three fold demonstrating this immunoglobulins' negative regulatory effect on this subset of B lymphocytes. However the local tissue homeostasis of these mice are unaffected. This may be

due to uptake of apoptotic bodies by the increased numbers of B-1 cells. In summary, the emerging role of B-1 cells and also perhaps the recently discovered IL-10 producing B-regulatory cells has potentially important implications in the regulating of both acute inflammation as well as promoting inflammatory resolution (Yang, Sun et al. 2010).

1.5 Mediators of resolution

Acute inflammation that is characterised by the movement of leukocytes from the blood into the microenvironment is facilitated by the release of pro-inflammatory cytokines and chemotactic mediators (Table 1.1). The focus of this thesis is the resolution potential of lipid mediators and adenosine which will be discussed in depth below:

1.5.1 Arachidonic acid metabolism by cyclo-oxygenase

Arachidonic acid [AA], released from the plasma membrane phospholipids via the action of calcium dependent phospholipase A2 (Figure 1.4). It is then converted to prostaglandin D_2 and H_2 sequentially by prostaglandin synthase also known as cyclooxygenase [COX]. PGH_2 formed from by the action of COX is then converted to prostaglandins [PGD_2, PGE_2 and $PGF_{2\alpha}$], thromboxane and prostacyclin by specific synthases.

There are three isoforms of Cyclooxygenase [COX], formerly known as prostaglandin G/H synthase [PGHS]. The first, COX-1 is constitutively expressed in most tissues and synthesises prostaglandins at low levels to maintain physiological function (Smith, Garavito et al. 1996). COX-2 is inducible in response to inflammatory and mitogenic stimuli (Hla and Neilson 1992; Sirois and Richards 1992) but is constitutively expressed in the forebrain (Kaufmann, Worley et al. 1996), macula densa of kidneys (Harris, McKanna et al. 1994) and in un-stimulated endothelial cells ex vivo (Grosser, Fries et al. 2006) and adjacent epithelial cells of the cortical thick ascending limb of the kidney (Harris, McKanna et al. 1994; Kaufmann, Worley et al.

1996). Both COX-1 and 2 share similar structural properties including a hydrophobic substrate specific tunnel. The COX-2 tunnel is more accommodating as it has a side pocket and therefore broader substrate recognition (Rajakariar, Yaqoob et al. 2006). It was observed that there was a rapid induction of COX-2 during inflammation accounting for the prostaglandin production, and the structural difference from COX-1 led to the discovery of drugs that selectively inhibit COX-2, which were marketed as being anti-inflammatory but without the gastric toxicity, latter implicated to the inhibition of COX-1. These selective COX-2 inhibitors (coxibs), celecoxib, rofecoxib and valdecoxib were approved by the US Food and Drug Administration (FDA) on the basis of small clinical studies in a period during which there were increasing evidence of the role of COX-2 in mediating inflammatory resolution. Furthermore, even though relatively small studies supported the gastro protective properties of selective COX-2 inhibitors, there were five large randomised controlled trials that demonstrated an increased risk of death from myocardial infarction and stroke in patients treated with coxibs leading to withdrawal of many compounds in this class. (Grosser, Fries et al. 2006). This may be explained by the observation that prostacyclin (PGI_2) production is mediated by increased expression of COX-2 in endothelial cells, its role of limiting platelet aggregation, as well as the lack of inhibition of pro-platelet aggregator TXA_2 production. Therefore, compared to traditional non selective non steroidal anti inflammatory drugs (NSAIDs), there is an imbalance between PGI_2 and TXA_2 in favour of increased expression of the latter leading to enhanced platelet aggregation (Grosser, Fries et al. 2006).

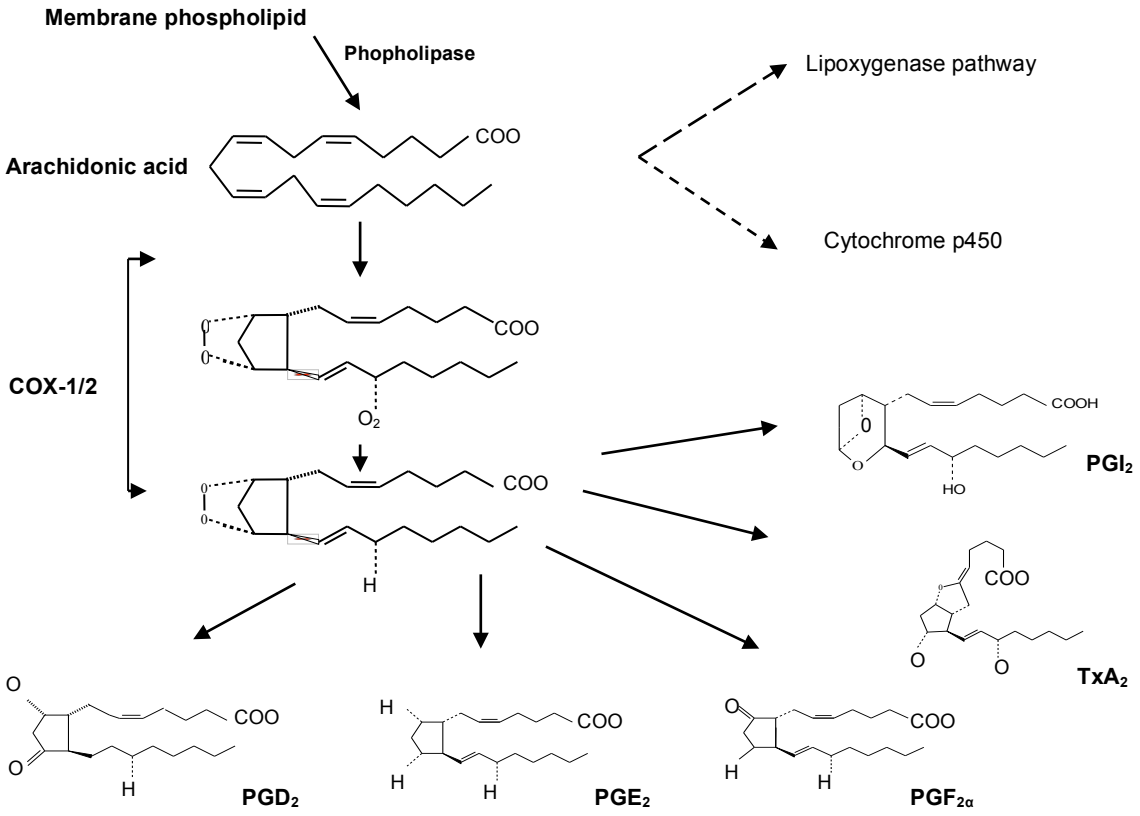


Figure 1.4 The Prostaglandin pathway-Synthesis of prostanoids by cyclo-oxygenase (COX) following the catalytic release of arachidonic acid from membrane phopholipids by by calcium dependent phospholipase A2. PGH_2 is metabolised to PGD_2, E_2, $F_{2\alpha}$, Thromobxane [TxA_2] and prostacylin [PGI_2] by specific synthases. Alternatively, arachidonic acid can be metabolized by lipoxygenase [5-, 12- & 15-] or cytochrome p450 to leukotrienes, lipoxins and hyroxyl/epoxyeicosatetraenoic fatty acids.

COX-3, a splice variant of COX-1 whose inhibition by acetaminophen is thought to promote the latter's anti-pyretic and analgesic effects (Ayoub, Botting et al. 2004). However the expression of acetaminophen sensitive COX-3 at a protein level in humans has been questioned (Schwab, Schluesener et al. 2003).

Even though the focus of this thesis is the resolution friendly mediators of the cyclo-oxygenase pathway, there are alternative ways in which arachidonic acid can be metabolized which include lipoxygenase [5-, 12- & 15-], cytochrome p450, leukotrienes, lipoxins and hyroxyl/epoxyeicosatetraenoic fatty acid (Figure 1.5) (Samuelsson, Dahlen et al. 1987).

As mentioned previously with the onset of resolution, there is a 'class switch' in the lipoxygenase pathway from pro-inflammatory leukotrienes that mediate increased vascular permeability and influx of leukocytes to site of injury to that of lipoxins (Serhan and Savill 2005). One of the molecules extensively studied is LXA_4 that has been shown to inhibit PMN chemotaxis, endothelial transmigration of granulocytes and reduce PMN mediated vascular permeability (Serhan, Hamberg et al. 1984). Lipoxins also enhance movement of monocytes to sites of inflammation and mediate monocyte-derived macrophage phagocytosis of apoptotic PMNs necessary to promote resolution (Maddox and Serhan 1996).

Following liquid chromatography tandem mas spectrophotometric assessment of exudates from inflamed dorsal air pouch model, another group of resolution mediators that is influenced by COX were discovered. They are resolvins and protectins, that are converted from omega-3 polyunsaturated fatty acids; eicosapentaenoic acid (EPA) and docasahexanenoic (DHA) respectively (Serhan, Hong et al. 2002). Endothelial cells in the presence of non-selective COX inhibitor aspirin, EPA is converted to resolvins that inhibit PMN migration enabling resolution (Bannenberg, Chiang et al. 2005).

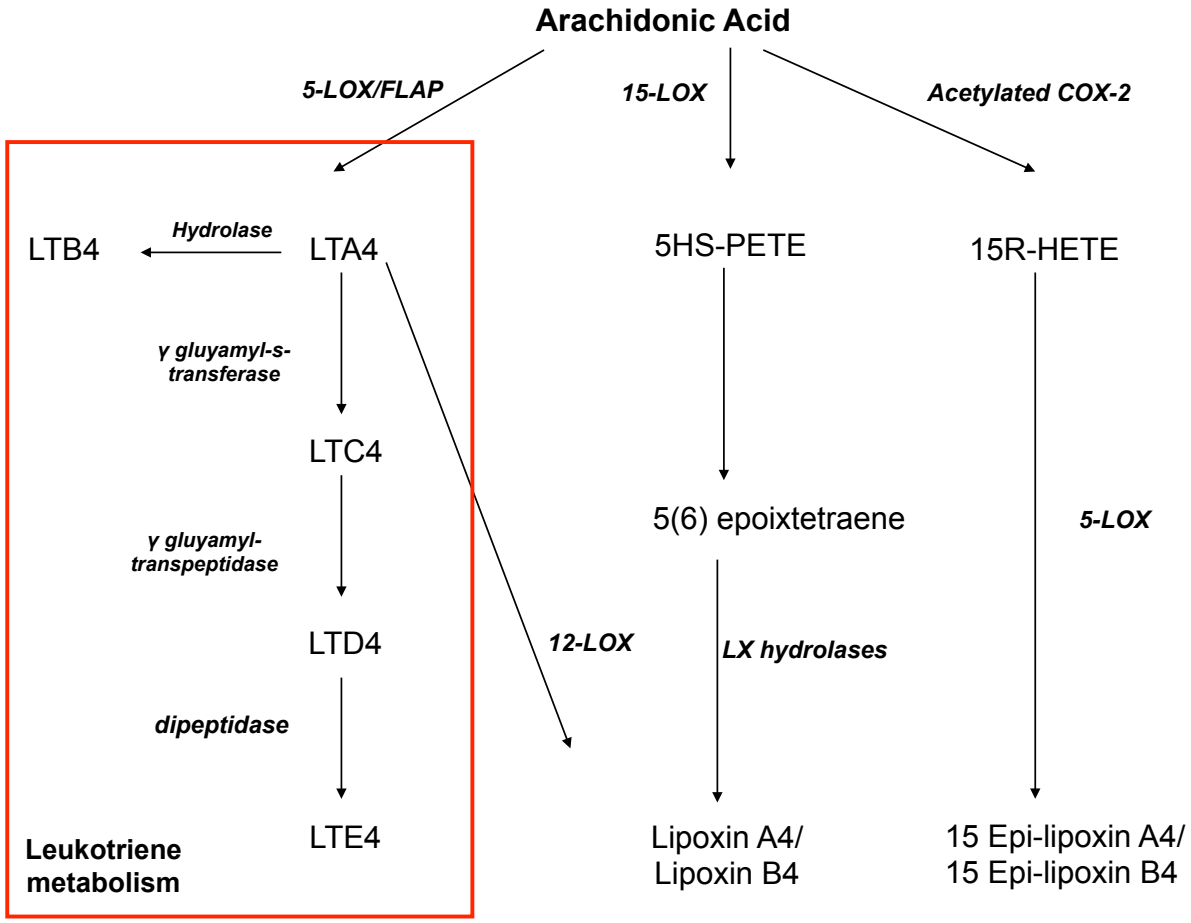


Figure 1.5 Lipoxigenase pathway- Leukotriene and lipoxin metabolism. LOX- Lipoxygenase. FLAP- 5-LOX Activating Protein. LT- Leukotriene. HPETE- hydroperoxyeicostetraenoic acid. HETE- hydroxyeicosatetraenoic acid. The red box is the leukotriene arm of the lipoxygenase pathway.

1.5.1.1 COX and inflammation

1.5.1.1.1 COX-1

As mentioned previously, COX-1 is constitutively expressed in most tissues and generates prostaglandins to maintain normal physiological functions. In cultured 3T3 cells, a fibroblast cell line, COX-1 was expressed at constant but low levels throughout the cell cycle. However to term COX-1 as *constitutive* whereas COX-2 being *inducible* is an oversimplification as COX-1 levels can be down-regulated in endothelial cells by acidic fibroblast growth factor and up-regulated in mast cells following response to stem cell factor and dexamethasone (Smith, Morrow et al. 1993; Kaufmann, Worley et al. 1996).

In the rat carrageenin-induced pleurisy model, with the use of more selective COX-1 inhibitors piroxicam and aspirin were shown to have greater and more prolonged anti-inflammatory effects than selective COX-2 inhibitors NS-398 and nimesulfide in the early stage [2 and 6 hours] of inflammation (Gilroy, Tomlinson et al. 1998). Also inflammatory models in COX-1 deficient mice provided further evidence to support its pro-inflammatory role. For instance, oedema induced by arachidonic acid was inhibited by 70% in knockout mice compared with wild-type mice (Langenbach, Morham et al. 1995). Further support for the pro-inflammatory role of COX-1 induced prostaglandin synthesis is based on the differential inflammatory response of both COX-1 and COX-2 deficient mice. Triphorbol myristate acetate response in ear swelling and carrageenin-induced paw oedema was similar in both COX-2 knockout mice and wild type mice suggesting that the constitutively expressed COX-1 isoform that is still present in both groups is synthesizing sufficient amount of prostaglandins for inflammation to proceed.

1.5.1.1.2 COX-2

In the seminal paper on the phenotype of COX-2, mice lacking this enzyme (COX-$2^{-/-}$) spontaneously developed suppurative peritonitis with multiple adhesions amongst the abdominal organs consistent with chronic inflammation (Morham, Langenbach et al. 1995). Therefore, it became apparent that perhaps some of the COX-2 metabolites were also responsible for the stop signals necessary for switching off inflammation. In a resolving acute lung injury model, selective COX-2 inhibition led to a rise in leukocyte counts in the broncho-alveolar lavage. In the gastro-intestinal system, COX-2 expression was up regulated in a rat ischaemia-reperfusion model and also following induction of colitis (Maricic, Ehrlich et al. 1999). Furthermore, haemorrhagic gastric injury significantly worsened with a selective COX-2 inhibition. During colitis, inhibition of COX-2 lead to worsening of colitis with intestinal perforation. This may

partly explain why the COXibs only reduced the rate of gastric ulceration by only 50% as it plays a protective role against luminal irritants (Wallace and Devchand 2005).

Consistent with the above findings, the anti-inflammatory role of COX-2 was further elucidated in a carrageenin induced pleurisy model in rats (Gilroy, Colville-Nash et al. 1999). In this model there were two peaks of COX-2. First in the early phase the rise in COX-2 was associated with production of the pro-inflammatory prostaglandin PGE_2. In the later phase of mononuclear cell dominated inflammation there was a 3-fold greater second peak of COX-2 coincident with the resolution phase latter mediated by another PGH_2 metabolite PGD_2 and its dehydration product $15deoxy\Delta^{12\text{-}14}PGJ_2$ (15d-PGJ2) (Gilroy, Colville-Nash et al. 1999). Both the non-selective COX-1/COX-2 inhibitor and the selective COX-2 inhibitor, NS-398, inhibited the acute inflammatory response, but impaired resolution with worsening inflammation 48 hours following induction of inflammation with reduced PGD_2 and 15-d-PGJ_2. The role of this cyclopentenone prostaglandin and its product 15-d-PGJ_2 will be discussed in detail in the following section (1.5.1.2.2 PGD_2 and 1.5.1.2.3 $15deoxy\Delta^{12\text{-}14}PGJ_2$).

1.5.1.2 COX derived mediators of inflammation and resolution

1.5.1.2.1 PGE_2

In the acute phase of carrageenin-induced pleurisy, there is an exaggerated release of PGE_2 formed from PGH_2 mediated by PGE synthase (PGES) (Gilroy, Colville-Nash et al. 1999). There are three isoforms- cytosolic PGES, membrane PGES 1&2 [mPGES-1 & 2]. The expression of these enzymes is up regulated by many pro-inflammatory cytokines including IL-1β and TNF-α. In the study of Astiz et al, levels of prostaglandin E_2 when measured in patients with sepsis or septic shock was significantly elevated compared to normal controls (Astiz, Saha et al. 1996). In mice deficient of mPGES-1, there is no PGE_2 production from peritoneal macrophages

stimulated with LPS and pain as assessed by ascetic acid writhing is significantly reduced (Kamei, Yamakawa et al. 2004). PGE_2 acts via the EP receptor, which has four subtypes [EP 1-4] (Andreasson 2010). The EP-2 and EP-4 receptors increase cAMP levels via activation of adenylate cyclase that further increases mPGES-1 expression in a positive feedback manner (Inoue, Takamori et al. 2002; Kojima, Kato et al. 2005). In a rat acute lung injury model caused by LPS, PGE_2 levels in brocho-aleveolar lavage was increased together with enhanced ICAM-1 expression on the vascular endothelium (Alba-Loureiro, Martins et al. 2004). In IL-1β stimulated human synovial fibroblast cultures, PGE_2 was shown to regulate the production of pro-inflammatory IL-6 and pro-angiogenic VEGF, as well as increasing EP 2 & 4 receptor mRNA expression (Inoue, Takamori et al. 2002). In the absence of IL-1β however, the ability of PGE_2 to produce IL-6 and VEGF was low suggesting the role of PGE_2 to be a promoter rather than a mediator of inflammation. In response to TNF-alpha, PGE_2 levels rose acutely followed by the anti-inflammatory lipoxin A4 [LXA4] formed by the conversion of AA by 15-lipoxigenase [15-LO] in a mouse air pouch model (Levy, Clish et al. 2001). PGE_2 has also been shown to mediate the expression of 15-lipoxigenase and inhibit the production of the pro-inflammatory LTB4 (Levy, Clish et al. 2001). Therefore, COX-2 derived PGE_2 not only promotes acute inflammation, but also modulates it by switching on pro-resolution factors. In an allergic pleuritis model in which both PGE_2 and LXA4 levels were raised in the pleural exudates, pre-treatment with a PGE_2 analogue, misoprostol, significantly reduced the duration of inflammation with no effect on the magnitude of the response (Bandeira-Melo, Serra et al. 2000).

1.5.1.2.2 PGD_2

In pursuit of the COX-2 derived pro-resolution factors, as mentioned previously, in the rat carragenin-induced pleurisy model, second peak of COX-2 activity during inflammatory resolution was not associated with a rise in PGE_2 but another PGH_2 metabolite, PGD_2 and its dehydration product 15deoxy$\Delta^{12\text{-}14}PGJ_2$ (15d-PGJ_2) (Gilroy, Colville-Nash et al. 1999).

There are two forms of PGD_2 synthase [PGD_2S]. The Lipocalin PGD_2S [L-PGD_2S], a 26 kDa protein is expressed in the central nervous system, testes, vascular endothelial cells, intimal smooth muscle cells and cardiomyocytes (Urade and Eguchi 2002). L-PGD_2S has been linked to diabetes, renal injury associated with hypertension and in patients with coronary artery disease (Hirawa, Uehara et al. 2002; Miwa, Takiuchi et al. 2004; Ragolia, Palaia et al. 2005). The haematopoietic PGD_2S [h-PGD_2S], also a 26 kDa cytosolic protein is responsible for conversion of PGH_2 to PGD_2 at sites of inflammation, within the central nervous system and in the testes. H-PGD_2S is expressed in mast cells, antigen presenting cells and T_h2 cells (Kanaoka and Urade 2003). In the pleurisy model, selective COX-2 inhibition during the resolution phase led to suppression of PGD_2 levels and the concomitant rise in exudates volumes and leukocyte numbers which was reversed with the addition of PGD_2 or 15d-PGJ_2 (Gilroy, Colville-Nash et al. 1999). The latter, a metabolite of PGJ_2 itself a formed by spontaneous dehydration of the cyclopentane ring of PGD_2 (Fitzpatrick and Wynalda 1983).

PGD_2 binds to and activates two G-protein coupled receptors DP1 (Narumiya, Sugimoto et al. 1999) and CRTH2 (Chemoattractant Receptor-homologous molecule expressed on T_h type 2 cells) (Hirai, Tanaka et al. 2001), which is also known as DP2, has emerged as an important factor released by mast cells during an acute asthmatic attack (Kabashima and Narumiya 2003). In adaptive immunity, dendritic cells that are antigen presenting cells can initiate immune responses with captured antigen when they have migrated to draining lymph nodes. PGD_2 has been shown to prevent the movement of epidermal Langerhans cells to the draining lymph nodes, when FITC labelled OVA were administered intra-tracheally to BALB/c mice. This inhibition of APC migration, were by the agonism of the DP1 and not the CRTH2 receptor (Angeli, Staumont et al. 2004). Whereas PGD_2 has an anti-inflammatory phenotype by the activation of the DP1 receptor, the opposite is true in its action on the CRTH2 receptor. The

activation CRTH2 receptor in the airways of rats resulted in eosinophilic inflammation (Hirai, Tanaka et al. 2001). PGD_2 has also been shown to induce pro-inflammatory Th2 type cytokines IL-4, IL-5 and IL-13 via its action on CRTH2 receptor but with no rise of the anti-inflammatory IL-10 (Xue, Gyles et al. 2005). However, it has been shown in our laboratory that in a delayed type hypersensitivity model, though lymphocytes isolated from $hPGD_2S$ knockout mice showed hyper proliferation and increased IL-2 levels these pro-inflammatory effects were not rescued by either selective DP1 or CRTH2 agonists (Trivedi, Newson et al. 2006).

A direct role of PGD_2 in innate inflammatory resolution via its G-coupled receptors requires further investigation. In a study using a murine air pouch model that mimics monosodium urate monohydrate crystal induced gouty arthritis, transfection of $hPGD_2S$ into C57/BL/6 fibroblasts led to persistent increased levels of PGD_2 and reduced PGE2 levels with concomitant reduction of cellular infiltration with crystal induced acute inflammation being inhibited (Murakami, Akahoshi et al. 2003). As in the air pouch model, PGD_2 is also elevated at the early stages of chemically induced rat colitis, and similar to the pleurisy model there is a substantial increase in PGD_2 in the absence of a rise in PGE_2 during the resolution phase (Zamuner, Warrier et al. 2003). In humans this is supported further by the up regulation of PGD_2 in patients in long term remission from ulcerative colitis (Vong, Ferraz et al. 2010).

1.5.1.2.3 15deoxy$\Delta^{12\text{-}14}PGJ_2$ (15d-PGJ_2)

In a carrageenin-induced pleurisy at 48 h after irritant injection, the second peak of COX-2 protein expression coincident with resolution of the inflammation, late influx of mononuclear cells was also associated with a rise of 15d-PGJ_2. Treatment with COX-2 selective inhibitors from 24 h to 48 h post-injection of irritant, i.e. during the resolving phase, resulted in a prolongation of the inflammatory response while concomitant administration of COX-2 inhibitors orally and therefore rescuing the depleted PGD_2 and cyclopentenone prostaglandins

locally, restored resolution (Gilroy, Colville-Nash et al. 1999). As mentioned above, PGD_2 undergoes spontaneous dehydration within its cyclopentane ring to $\Delta^{12}PGJ_2$ and then 15d-PGJ_2 which is characterized by the presence of a reactive α,β-unsaturated ketone in the cyclopentenone ring (Straus and Glass 2001). The reactive ketones contain an electrophilic centre which makes prostaglandins to undergo addition reactions termed 'Michael addition' with nucleophiles such as free sulfhydryl group of cysteine residues located in reduced glutathione or cellular proteins (Fukushima 1992), for example the high affinity binding of 15d-PGJ_2 to intracellular PPARγ (Ide, Egan et al. 2003).

Peroxisome proliferator-activated receptor [PPAR], a family of transcription factors that regulate gene expression of enzymes associated with lipid homeostasis, inflammation cell proliferation and malignancy. PPAR family consist of three isoforms- PPAR-α, PPAR-β/δ and PPAR-γ (Zhu, Qi et al. 1995; Fajas, Debril et al. 2001). All three contain a central DNA binding domain that recognizes DNA sequences known as PPAR response elements. PPAR-γ was originally thought to be expressed only in adipocytes and hepatocytes, but was later found in many other cell types including macrophages/monocytes, myocytes, fibroblasts and human bone marrow precursors (Greene, Blumberg et al. 1995; Braissant, Foufelle et al. 1996). Though resident peritoneal macrophages express low levels of PPARγ, its expression is induced to high levels in activated macrophages. Binding of 15d-PGJ_2 leads to its translocation from the cytoplasm to the nucleus and inhibition of pro-inflammatory AP-1, NF-κB and STAT1 transcription (Ricote, Li et al. 1998).

15d-PGJ_2 has also been shown to induce responses by inhibiting multiple steps of the pro-inflammatory NF-κB pathway independent of PPARγ (Straus, Pascual et al. 2000). NF-κB is an important mediator of innate immune responses, which is responsible for induction of pro-inflammatory genes such as cytokines, chemokines and adhesion molecules. NF-κB is usually in

its inactive state by being bound to the inhibitory protein IκB in the cytoplasm. On activation, IκB is phosphorylated by IκB kinase [IKK] leading to proteasomal degradation. This allows the active components of NF-κB, p50 and p65 to translocate to the nucleus to regulate gene expression. 15d-PGJ_2 has been shown independent of PPARγ to impact on the NF-κB pathway, including inhibition of IKK and direct inhibition of NF-κB binding by alkylation of the cysteine residue located in NF-κB binding domain (Straus, Pascual et al. 2000).

In a carrageenin induced pleurisy model in rats, 15d-PGJ_2 brings about inflammatory resolution by leukocyte apoptosis (PMNs and macrophages) as determined by Annexin/PI staining and TUNEL methods (Gilroy, Colville-Nash et al. 2003). It is likely 15d-PGJ_2 brings about its pro-apoptotic effects and therefore aiding resolution via the inhibition of the NF-κB pathway (Gilroy, Lawrence et al. 2004). Inhibition of NF-κB has been shown to significantly increase granulocyte apoptosis in response to TNF-α and then allowing the clearance of apoptotic cells by phagocytic macrophages and for resolution to occur (Hallett, Leitch et al. 2008). 15d-PGJ_2 has similar effects on eosinophil survival and may play a role in the resolution of allergic inflammation. Apart from inducing leukocyte apoptosis, 15d-PGJ2 can also inhibit monocyte trafficking, regulate macrophage activation and pro-inflammatory gene expression (Jackson, Parhami et al. 1999; Pasceri, Wu et al. 2000; Zhang, Wang et al. 2001; Lawrence 2002).

Central to the role of COX-2 derived cyclopentenone prostaglandins in the resolution of inflammation is whether it plays a role in normal physiological conditions. PGD_2 and 15d-PGJ_2 have been demonstrated in biological samples using EIA kits developed by Cayman Chemical Company Ltd. However the 15d-PGJ_2 assay has been criticized for having high background levels and a more recent kit by R&D systems has become more popular. However the best method for the measurement of 15dPGJ_2 is liquid chromatography–mass spectrometry–mass spectrometry [LC-MS-MS]**.** Using this technique Bell-Parikh et al have failed to demonstrate

significant levels of 15d-PGJ_2 in sites of inflammation in humans (Bell-Parikh, Ide et al. 2003). However it was measured in the synovium of patients with rheumatoid arthritis a chronic autoimmune inflammatory model. It is conceivable that the absence or depressed levels of 15d-PGJ_2 may be a reflection of the 'non-resolving' nature of the inflammatory response and its profile in an *in vivo* resolving model of inflammation require investigation.

1.5.2 Adenosine

Adenosine is a purine nucleoside, a product of ATP degradation that rises by up to 200 fold in response to inflammation and hypoxia. It acts via A1, A2A, A2B and A3 receptors (Fredholm, AP et al. 2001; Eltzschig, Sitkovsky et al. 2012). With respect to inflammation most attention has been addressed to the A2A receptor, which has potent anti-inflammatory properties.

Adenosine is produced by the breakdown of ATP as well as AMP via the action of membrane bound ecto-apyrases such as CD39 and CD73, the latter being the rate limiting step. These enzymes are abundant in lymphocytes and endothelial cells and strategically located in the inflammatory cascade to modulate the acute inflammatory response. Adenosine has a short half-life and is broken down to inosine by adenosine deaminase (ADA), which is both cytosolic and membrane bound (Figure 1.6). Inosine, when finally broken down to uric acid which is excreted in the urine (Kumar and Sharma 2009). The breakdown of the 'active' adenosine to inosine is a necessary event, as persistence of this powerful anti-inflammatory mediator will limit the ability of the host to fight the offending agent leading to persistent and on going tissue damage.

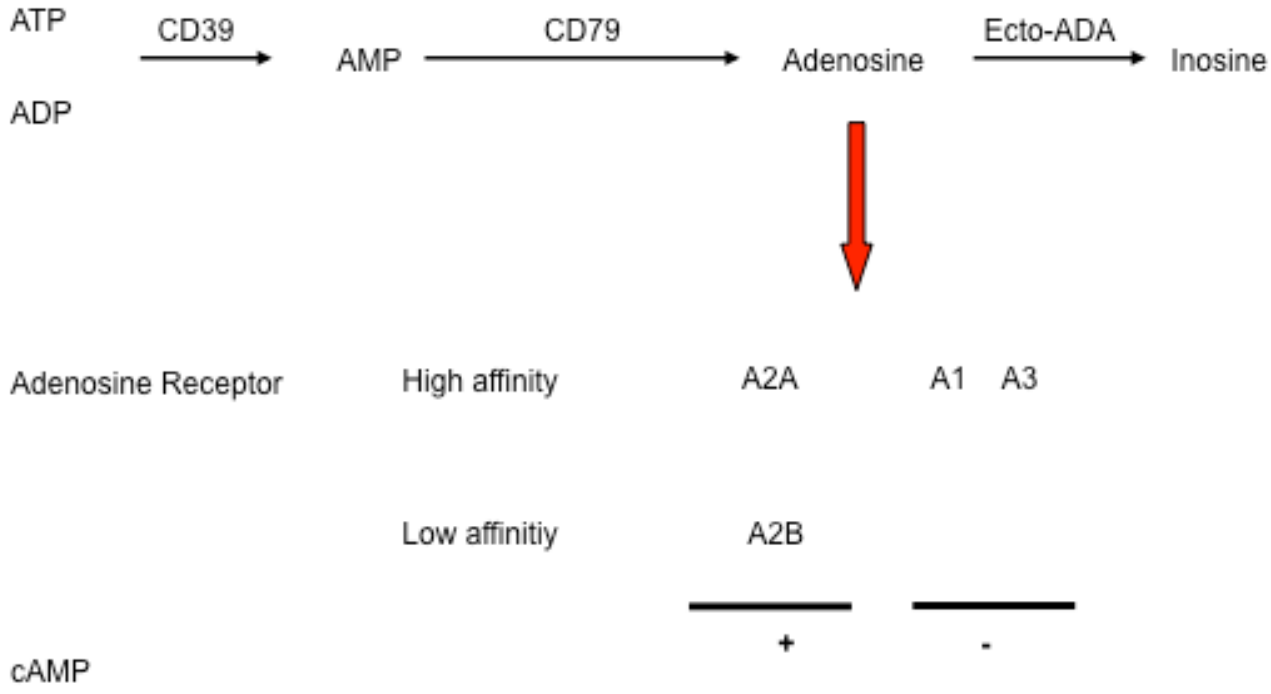


Figure 1.6 Adenosine metabolism- Adenosine production by the breakdown of ATP/ AMP by ecto-apyrases such as CD39 and CD73. Adenosine is then broken down to inosine by adenosine deaminase (ADA).

1.5.2.1 **A2A receptor**

Adenosine binding to A2A receptor leads to stimulation of the G protein coupled adenylyl cyclase (AC) leading to rise in intra-cellular cyclic AMP (cAMP) with activation of down-stream protein kinase A (PKA), cyclic nucleotide gated ion channels and exchange proteins directly activated by cAMP (EPAC) that in turn mediate its anti-inflammatory effects (Thiel, Caldwell et al. 2003). It exerts its action by inhibiting pro-inflammatory signalling pathways e.g. the NF-κB and Janus kinase/signalling transducer and activator of transcription (JAK/STAT) pathways respectively. A family of cAMP phosphodiesterases opposes this by hydrolysing cAMP to 5'AMP, leading to signal termination (Milne and Palmer 2011).

The A2A receptor is expressed on PMNs, Monocytes, T lymphocytes and NK cells. In innate immunity the effect of adenosine via the A2A receptor leads to decreased recruitment to sites of

inflammation due to decreased endothelial cell adherence (Hasko, Linden et al. 2008). This is due to cAMP and PKA-dependent down regulation of VLA-4 ('Very Late Angtigen-4) that interacts with VCAM-1. Also A2A activation leads to inhibition of phagocytosis and decreased production of ROS. A2A receptor activation in macrophages leads to direct reduction of pro-inflammatory mediators such as TNFα, IL-12 or increased anti-inflammatory cytokine IL-10 (Mosser and Edwards 2008). The latter further blocks pro-inflammatory IL-6 as well as the chemokine MCP-1. These actions can occur by both cAMP and PKA dependent and PKA independent EPAC dependent mechanisms (Milne and Palmer 2011). Adenosine also plays an anti-inflammatory role in the adaptive arm of immunity via its action on various subsets of T lymphocytes that have been summarised in Table 1.2 shown below (Ernst, Garrison et al. 2010; Milne and Palmer 2011).

CD4	T_h1 and T_h2	Suppression of proliferation and maturation Inhibition of pro-inflammatory cytokine production
	Tregs	Up regulation of FOXP3 expression
CD8		Suppression of cytolytic activity Inhibition of pro inflammatory cytokine production

Table 1.2 Effect of adenosine on T cell subsets

In contrast to the A2A receptor, the A2B receptor is of low affinity to adenosine and therefore requiring greater tissue concentrations of adenosine for activation. Like A2A, it is also coupled to the $G_{s\alpha}$ subunit of adenylyl cyclase leading to increased cAMP levels on activation. In spite of

A2B being of low affinity, adenosine levels rise to sufficient levels during hypoxia and inflammation to activate this receptor, which also has anti-inflammatory properties that has been shown to modulate inflammation [e.g.: colitis] and renal ischaemia-reperfusion injury (Aherne, Kewley et al. 2011).

Both the A1 and A3 receptors similar to A2AR, have a high affinity to adenosine. Activation of the A1 receptor leads to inhibition of cAMP production. Its activation has a depressant effect on the conduction system in the heart and therefore explains the anti-arrhythmic properties of adenosine. A3 receptor however also has potent anti-inflammatory properties and a selective agonist CF-101 is being investigated as a disease-modifying agent in rheumatoid arthritis (Varani, Padovan et al. 2010).

1.6 Models of Chronic inflammation

Resolution of inflammation occur when the acute pro-inflammatory signals are balanced by 'stop' signals such as COX-2 derived PGD2, its cyclopentenone metabolites, lipoxins, resolvins which lead to removal of dead cells and facilitate the exit of inflammatory cells. The failure of the 'stop' signals to counterbalance the triggers for acute inflammation, lead to persistent oedema, abscess formation tissue scarring and deformity for example, chronic deforming arthropathy in rheumatoid arthritis. This is further illustrated by two clinical examples, encapsulating peritoneal sclerosis (EPS), a potentially fatal complication in patients with End Stage Renal Disease (ESRD) undergoing peritoneal dialysis (PD), and Chronic Granulomatous Disease (CGD), an inherited condition due to impaired phagocytosis.

1.6.1 Peritoneal Dialysis (PD) and encapsulating peritoneal sclerosis (EPS)

Peritoneal dialysis is a widely practiced form of renal replacement therapy in patients with end stage renal failure. In an audit in London the prevalence is 28% (Davenport 2009) though the number of patients on this modality is falling (Castledine, Casula et al. 2012). For a patient requiring dialysis, it is often the first line modality, which affords many advantages, principally because it is a home-based treatment. Also worldwide, it has provided the means of managing patients who would otherwise have been denied treatment due to resource limitations. This method involves the surgical placement of intra-abdominal catheter and the use of the peritoneal lining as a dialysis membrane. Dialysis consist of infusion of fluid rich in either glucose or glucose-based polymers and the movement of uraemic toxins and water down an osmotic gradient. Once steady state has been achieved the peritoneal fluid is removed and fresh dialysate is infused. There are two predominant forms of peritoneal dialysis. Continuous Ambulatory PD [CAPD], this involves multiple exchanges during the day (usually three) followed by an overnight dwell. Automated PD (APD) is an automated form of therapy in which a machine cycler delivers up to 6 exchanges while the patient is asleep. There may be an additional 12 to 15 h daytime dwell depending upon the adequacy of dialysis and volume status of the patient.

1.6.1.1 Peritonitis

Peritonitis is the most common and significant complication of peritoneal dialysis (Mactier 2009). It accounts for up to 35% of hospital admissions of patients on PD and remains the main reason for the switch to hospital based haemodialysis and mortality(Boudville, Kemp et al. 2012).

Peritonitis is defined as a patient presenting with two or more of the following:

- Clinical symptoms and signs- Abdominal pain, nausea and vomiting, tender abdomen on palpation
- Cloudy dialysate
- >100 leukocytes per mm^3 on microscopy

In most cases the causative organism is identified, the majority of which is due to pathogenic bacteria (Table 1.3).

In up to 25% of cases the culture may be negative (Davenport 2009), which may be due to the culturing of the effluent very early in the course of peritonitis when the colony numbers are low, antibiotic contamination or inappropriate culture technique.

Treatment of peritonitis involves empirical antibiotic therapy based on the known incidence of different pathogens followed by treatment based on the cultured organism and antibiotic sensitivities. Resolution of the inflammation occurs in 90% of the episodes with appropriate antibiotic therapy. In the remainder the catheter is removed and patients transferred to haemodialysis. Catheter removal rates are higher with *S. aureus* and gram-negative infections. In addition, the removal of the PD catheter is mandatory if the infection is due to Pseudomonas or fungi. Failure of resolution is greater in patients with culture negative peritonitis and those who relapse soon after a previous treated episode.

	Episodes/patient-year
Gram-positive	
Staphylococcus epidermidis	0.17-1.04
Staphylococcus aureus	0.09-0.15
Streptococcus	0.04-0.14
Enterococcus	0.01-0.04
Other Gram-positive	<0.01-0.02
Gram-negative	0.09-0.24
Fungal	<0.01-0.07
Mycobacterial	<0.01
Sterile	0.10-0.20

Table 1.3 Organisms responsible for peritoneal dialysis peritonitis. Adapted from Gokal *et al* Textbook of peritoneal dialysis 2nd Edition Kluwer academic publishers 2000

Peritonitis in patients on peritoneal dialysis affords an imperfect but the closest human equivalent to study the changes in cytokines in both acute resolving and non-resolving inflammation as inflammatory exudate samples can be obtained during the course of inflammation. The diversity of patients, varied time of presentation and collection of PD effluent mean obtaining samples for analysis is practically difficult. Therefore the available information in the literature is limited. In a study by Lai et al, serial changes in cell and cytokine profiles was assessed in 35 patients with acute bacterial peritonitis and compared with the PD effluent of 76 patients with no infection (Lai, Lai et al. 2000). Even though clinically resolved the total leukocyte counts were significantly greater. Cytokine levels were measure at day 1, 3, 5, 10, 21 and 42. Peak levels of pro-inflammatory IL-1β, IL-6 and anti-inflammatory TGF-beta was found at day 1 with progressive decrease with resolution. However even at 6 weeks, the effluent of

infected PD patients the levels were significantly greater than in non-infected controls (Lai, Lai et al. 2000). In another study, the investigators recognized two groups of patients with peritonitis, one with a rapid response to treatment with a progressive increase in T cell CD4/CD8 ratio and another group with delayed response to therapy with a decrease in CD4/CD8 ratio (Wang, Lin et al. 2003). The authors suggested the pattern of T cell may influence both treatment and future *sequelae* (Wang, Lin et al. 2003; Griveas, Fleva et al. 2009).

1.6.1.2 Encapsulating peritoneal sclerosis

EPS is by far the most serious and devastating consequence of peritoneal dialysis (Chin and Yeun 2006). The affected patient may present with abdominal pain, vomiting and intermittent bowel obstruction leading to progressive malnutrition. The small intestine is bound or encapsulated by a thick fibrous layer. The prevalence varies amongst different clinical units, from 0.7% to 3.7%. Risk factors include duration on peritoneal dialysis treatment, recurrent and 'non-resolving' grumbling and severe peritonitis, poor biocompatibility of the dialysate and duration on peritoneal dialysis. EPS is not exclusively related to PD and can occur with autoimmune disease, beta-blocker therapy, intra-abdominal malignancy and exposure to talc. Research in this area has been hampered by the lack of reproducible animal models. A 'two hit' hypothesis has been proposed with the initial insult being peritoneal exposure to dialysate fluid (glucose, glucose degradation products, pH) in the context of uraemic state (Honda and Oda 2005). This leads to increased TGFβ and VEGF activity and peritoneal fibrosis that can occur over years. A 'second hit', i.e. peritonitis, withdrawal of peritoneal dialysis, transplantation can accelerate the pro-fibrotic process leading to adhesions, the hallmark of EPS. The progression of peritoneal sclerosis and EPS may be a consequence of the imbalance between pro and anti-inflammatory cytokines leading to accumulation macrophages and fibroblasts leading to a pro-fibrogenic state.

There is scant evidence for the role of resident lymphocytes in modulating both peritonitis and EPS. A recent study identified a population of effector memory T cells (T_{EM}) that were potentially protective against invading pathogens (Roberts, Baird et al. 2009). Furthermore in an animal model of peritoneal sclerosis, rosiglitazone, a PPARγ agonist, was able to ameliorate the degree of fibrosis (Sandoval, Loureiro et al. 2010). This was associated with increased $CD3^+$ T lymphocytes in particular $CD4^+$ $CD25^+$ $FoxP3^+$ cells as well as increased IL-10 levels. Another study of PD effluents from patients who previously had peritonitis showed a progressive decrease in the CD4/CD8 lymphocyte ratio that was associated with increased TGF-β1 expression and change in transporter status to a rapid equilibration with plasma for glucose, a risk factor for developing EPS (Wang, Lin et al. 2003).

1.6.2 Chronic granulomatous disease

Chronic granulomatous disease (CGD) is disease affecting the innate immune system characterised by impaired phagocytosis due to absence of superoxide generating NADPH oxidase (NOX). This makes patients with CGD susceptible to serious bacterial and fungal infections with an incidence of 1 in 200,000 births (Segal, Leto et al. 2000; Winkelstein, Marino et al. 2000). Over two thirds of CGD is X-linked recessive and therefore predominantly affects males. There are no known autosomal dominant cases.

NADPH is an enzyme complex which comprise of membrane bound b558 (consisting $gp91^{phox}$ and $p22^{phox)}$ and cytosolic $p47^{phox}$ and $p67^{phox}$ components (Holland 2010). CGD can manifest due to mutations in any of the four structural genes. Mutations in $gp91^{phox}$ account for 65% of clinical presentations (Holland 2010). The critical regulatory component for activation of NOX is $p40^{phox}$ and Rac. On cellular activation, the phosphorylated cytosolic components associate with $p40^{phox}$ and Rac, which then combine with $gp91^{phox}$ and $p22^{phox}$ that result in the activation of the NADPH complex. This leads to production of superoxide, a ROS, which in the

presence of superoxide dismutase is converted to hydrogen peroxide that is critical for microbial killing (Segal, Leto et al. 2000). The main sources of ROSs are phagocytic cells, namely PMNs and macrophages. In addition to direct microbicidal activity via superoxide and its metabolites, hydrogen peroxide, peroxinitrite, hypochloric acid, ROS also arrest pathogen survival and growth and activate the formation of neutrophil extracellular traps (NETs) (Kirchner, Moller et al. 2012).

The commonest infections in CGD patients are *Staphylococcus aureus, Burkholderia cepacia, Serratia marcescents, Nocardia* and *Aspergillus* (Segal 1996; Segal, Leto et al. 2000; Martire, Rondelli et al. 2008). Other infectious organisms associated with CGD being Salmonella and Tuberculosis. The overall survival from CGD has improved over time with the advent of co-trimaxazole prophylaxis and anti-fungal agents.

1.7 Objectives of this thesis

1.7.1 Hypothesis

1. **PGD2 and its cyclopentenone metabolite 15d-PGJ2 are essential for resolution of inflammation in mammalian model of acute resolving inflammation (Chapter 3)**

As has been the theme of this introduction to the thesis, resolution of inflammation is an active process. The work in our laboratory has been in dissecting the role of lipid mediators of the cyclo-oxygenase pathway in inflammatory resolution. I therefore investigated the role of PGD_2 and its downstream metabolite 15d-PGJ_2 in inflammatory resolution. To this end, a sterile resolving model of chemical peritonitis induced by a fungal product zymosan was used with inflammation induced in genetically modified mice lacking the $hPGD_2S$ and wild type controls.

As doubts were cast on the physiological significance of 15d-PGJ_2 in mammalian systems, this was measured by LC-MS-MS in selected time points of acute peritoneal inflammation.

2. Resolving phase lymphocytes promote resolution of peritoneal inflammation by a PGD2 dependent manner (Chapter 4)

During the course of the study, changes in lymphocyte numbers were observed, to be reduced in numbers during the acute phase followed by re-population during the resolution phase. Therefore lymphocytes during the course of peritonitis were characterised and the role of PGD_2 in modulating the cellular profile was also investigated

3. Reduced production of adenosine is responsible for the hyper-inflammatory phenotype in mice lacking the gp91 phox subunit of NADPH oxidase, a model of chronic granulomatous disease, during zymosan peritonitis and is due to decreased activation of the A2A receptor (Chapter 5)

In order to elucidate what happens in a non-resolving chronic innate model of inflammation with respect to both cellular components as well as mediators of resolution, mice lacking the $gp91^{phox}$ subunit of NADPH oxidase, a model of chronic granulomatous disease was investigated. There was no difference in PGE_2 and PGD_2 levels in knock out mice compared to wild types. Therefore, reduced expression of xanthines- adenosine and its metabolites were thought to play a role in the failure of resolution in CGD mice.

Chapter 2 Materials and methods

This chapter will describe all of the general methods used in this thesis as a whole. The design of individual experiments will be described in the results chapters. All materials unless mentioned otherwise were purchased by Sigma-Aldrich Chemical Co., UK.

2.1 Animal Husbandry

C57 black VI male mice [8 weeks old] were purchased from Charles River, Margate, Kent, UK. Three different genetically modified mice of C57BL/6 mouse strain were used for *in vivo* experimentation, $hPGD_2$ synthase knock out ($hPGD_2S^{-/-}$), RAG2 knockout ($RAG2^{-/-}$) and NADPH oxidase $gp91^{phox-/-}$ mice. Animal work was performed according to Home Office regulations [Scientific Procedures Act 1986].

2.2 $hPGD_2$ synthase knockout mice ($hPGD_2S^{-/-}$)

A breeding program for $hPGD_2$ synthase knockout mice was undertaken in the Biological Services Unit at the William Harvey Research Institute, Charterhouse Square, London. The mouse colonies were housed under standard conditions in a room with controlled lighting [lights on 08:00-22:00] in which the temperature was maintained at 21-23ºC and on a standard chow pellet diet with tap water *ad libitum*. The litters from the $hPGD_2$ synthase knockout colonies were genotyped as below and male knockout mice and littermate controls were used for the experiments. However when there was an excess of knockout and heterozygotes commercially purchased age matched C57BL/6 wild type mice were used.

2.2.1 Generation of H-PGD_2S gene-disrupted mice

$hPGD_2S$ gene-disrupted mice were generated through the standard gene targeting technology with mouse embryonic stem cells derived from the 129 strain and backcrossed to the inbred C57BL/6J strain 15 times. The strain was created at Osaka Bioscience Institute and Japan

Tobacco Inc. Pharmaceutical Frontier Research Laboratories. Figure 2.1 summarizes the development of the hPGD$_2$S knockout mice.

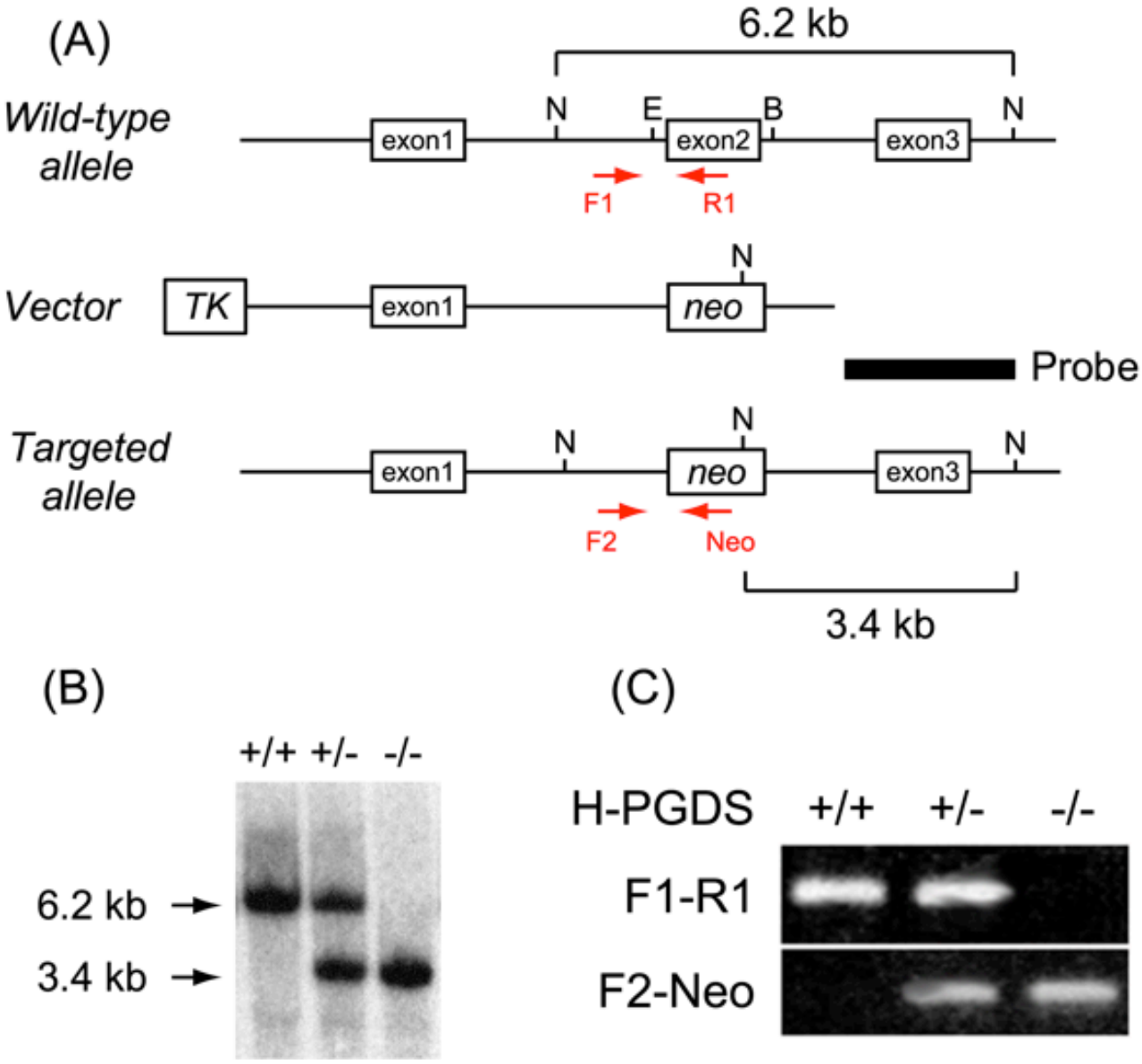


Figure 2.1 Structure of the wild-type H-PGDS allele (Top), the targeting vector (Middle), and the targeted allele (Bottom). The probe used for Southern blot analysis is shown as a thick line. Restriction sites: N, *Nco*I; E, *Eco*47III; B, *Bst*XI. TK, Herpes simplex virus thymidine kinase gene; neo, neomycin resistance gene. (B) Southern blot analysis of *Nco*I-digested tail DNA (10 μg/lane) from heterozygous offspring with the ^{32}P-labeled probe as shown in (A). (C) Genotyping of wild-type and H-PGDS knockout mice by PCR analysis. For genotyping for H-PGDS, primer F1 (5'-GAGTTGCTGCATCTGACCTTTC-3') and R1 (5'-TAGCGAATAATTTCGGCTCTTCC-3') were used for the detection of wild-type allele, and F2 (5'-AAGATCTGTCTTGTTGCGTACGCT-3') and Neo (5'-GTCCAGATCATCCTGATCGAC-5'), for that of the targeted allele.

2.2.2 Genotyping of mice

Genotyping was necessary to identify the knockout mice from heterozygote and wildtype mice. To this end mouse tissue (ear notch) was first digested and polymerase chain reaction (PCR) with the appropriate primers were undertaken.

2.2.2.1 Tissue Digestion

The mice were ear notched for identification and the ear tissue was used for genotyping. It was digested with 50 µL of digestion buffer overnight at 55ºC. Digestion buffer comprised of 0.1 mg/mL Gelatin, 100mM Tris pH 8, 500 mM KCl, 0.45% NP-40, 0.45% Tween-20, 500 µg/mL Proteinase K. The samples were spun briefly and then incubated at 94ºC for 10 min to inactivate the proteinase K followed by centrifugation at 958g for 5 min. 2 µL of supernatant was used for the PCR reaction.

2.2.2.2 Polymerase Chain reaction

PuReTaq^{TM}Ready-To-Go^{TM}PCR beads [Amersham] were used.

The primers were as follows:

Wild type allele	F1	5'-GAGTTGCTGCATCTGACCTTTC-3'
	R1	5'-TAGCGAATAATTTCGGCTCTTCC-3'
Knockout allele	F2	5'-AAGATCTGTCTTGTTGCGTACGCT-3'
	Neo	5'-GTCCAGATCATCCTGATCGAC-5'

The primers were diluted to 1 µM and 5 µL of the wild type and knockout primers were multiplexed with 13 µL of molecular grade water and added to the PCR beads. 2 µL of the digestion was then added. In the final volume of 25 µL each bead contained 2.5 units of puRe Taq DNA polymerase (GE healthcare, Buckinghamshire, UK), 10 mM Tris-HCl (pH 9), 200 µM

dNTPs, 50 mM potassium chloride and 1.5 mM magnesium chloride. The reaction conditions: initial denaturation at 94ºC for 5 min followed by 94 ºC 30 s/ 55 ºC 30 s/ 72 ºC 50 s for 35 cycles. Following the PCR reaction, the loading buffer [40% sucrose and methylene blue] was added [3 parts reaction mixture 1 part loading buffer]. Reaction was run on a 1% agarose gel with 0.001% Ethidium Bromide. A positive control from a previous batch of reaction and a negative control [no DNA sample] were included in each gel. Bands for wild type (WT) and knockout (KO) alleles were visualized with a UV transilluminator. Figure 2.2 shows the relative positions of wild type and knockout alleles.

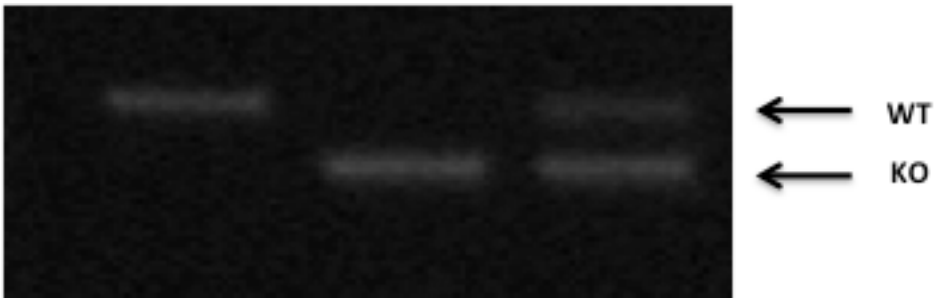


Figure 2.2 Bands of WT and KO alleles visulaised by UV transilluminator with the upper band corresponding to the WT allele and lower being the knockout allele.

2.3 Study of Peritonitis

Peritoneal inflammation was used as a model of innate inflammation to test the hypothesis generated in this thesis. Both a murine and human model of peritonitis was investigated.

2.3.1 Murine Peritonitis

Murine peritonitis was induced with the intraperitoneal administration of two different agents in the course of experiments. All experiments however were carried out with the i.p. administration of Zymosan Type A *Saccharomyces cerevisiae* [Sigma catalogue number Z4250] to induce an experimental sterile inflammation. However in the study assessing the role of re-populating

lymphocytes during resolution following zymosan peritonitis (detailed in chapter 4), group B streptococcus (GBS) was administered i.p. to induce peritonitis.

2.3.1.1 Preparation of Zymosan Type A for experimentation

Zymosan A is activated in order to induce an inflammatory response and this form is stored at -20ºC until use. One gram was placed in a conical flask with 200 mL of sterile PBS. The mixture is heated for 30 minutes with constant stirring until boiling point is reached. Once the suspension has cooled, it is then aliquoted into 20 tubes [10mL each] and centrifuged at 310g for 10 min at 4ºC. The supernatants were washed three times with sterile PBS. Pellets are then pooled sequentially until a single pellet of zymosan is left. The pellet is resuspended in 20 mL [ie: 1000 mg in 20 mL] and then aliquoted. The aliquots are centrifuged at 3000 rpm for 5 min and the pellets are frozen at -20ºC until use. On day of use the aliquot is allowed to defrost at room temperature for 30 min and then suspended in sterile PBS [pH 7.2] at a concentration of 2 mg/mL for use.

2.3.1.2 Preparation of Group B streptococcus (GBS) for experimental peritonitis\

The clinical GBS isolate, NCTC10/84 (serotype V) was grown in Todd Hewitt Broth (THB) without agitation at 37^{o}C to an OD_{600} of 0.4; equivalent to $1x10^{8}$ CFU/mL. Bacteria collected by centrifugation were washed with sterile PBS.

2.3.1.3 Induction of murine Peritonitis

Peritonitis was induced by a single injection of Zymosan A 1 mg in 0.5 mL of sterile PBS, pH 7.2. At selected time points the mice were killed by CO_2. The peritoneal cavity is then injected with 3 mL of sterile PBS followed by gentle massage to circulate the buffer within the peritoneal cavity. The peritoneum is then exposed and the lavage is transferred to 2mL eppendorf tubes

using a 3 mL Pasteur pipette. In the survival experiments described in chapter 4 during the resolution phase of zymosan peritonitis, mice were inoculated by i.p. injection with $5x10^7$ CFU in 0.3 mL of PBS.

2.3.1.4 Measurement of cell numbers

Cell number in the lavage was determined by staining of nuclei with Turks' solution (0.01% Crystal Violet, 3% Ascetic Acid). 900 µL of Turks'solution is added to 100 µL of cells from lavage. Cell number is then counted using a haemocytometer.

2.3.1.5 Trypsinization of peritoneal cavity

During the acute phase of peritonitis, as PMN numbers peak, lymphocyte numbers fall only to repopulate during the resolution phase. One hypothesis was that the peritoneal lymphocytes become adherent to visceral peritoneal lining. Therefore to determine the fate of peritoneal T and B cells, 4 h after zymosan injection, peritoneal cavities of mice were lavaged with sterile PBS to remove accumulated inflammatory cells. Then, 5 mL of pre-warmed 5% trypsin was then added to the peritoneal cavity of these for 10 min followed by an equal volume complete medium to acquire cells adhered to the peritoneal lining/greater omental lymphoid organ. Cells were then analysed for composition by flow cytometry.

2.3.2 Human Peritonitis

We considered peritonitis a complication of patients undergoing peritoneal dialysis, to be an ideal human model to investigate innate inflammation and resolution as effluent samples can be obtained at selected time points. Ethical approval [P/03/136A] for collection of human peritonitis samples was obtained from St. Bartholomew's & the Royal London Hospitals from end stage renal failure patients undergoing peritoneal dialysis. When patients develop peritonitis usually at

home, the effluent is brought in to hospital when they arrive to commence treatment. A sample is sent for microscopy, culture and the remainder is collected for experimentation. Less commonly the patient may have already been admitted to hospital for another reason when he or she develops peritonitis. The patient then commenced on antibiotic treatment and either sent home or admitted depending on the severity of symptoms. If the patient is sent home, they then return on day 3, 5, 10 and 15 when the overnight effluent is drained out and collected for experimentation. If admitted, the overnight dwell when drained out the following morning is used for experimentation.

Once the sample is collected, the cell numbers were counted as described above (2.3.1.4 Measurement of cell numbers). The cellular profile was assessed by specific fluorescent antibodies using flow cytometry as described below (2.4.1 Flow cytometry). The cell rich effluent was then centrifuged at 1500 rpm for 5 min and cell free supernatant was stored at -80°C until further use.

2.4 Analysis of leukocyte profile and function

2.4.1 Flow cytometry

The differential cell profile is analysed by flow cytometry using FACSCalibur and CellQuest software [BD Biosciences]. Following the cell count the appropriate volume of exudates containing 250,000 cells was centrifuged at 239g for 5 min, then washed with sterile PBS and re-suspended with 50 µL of PBS pH 7.2/1%BSA/20 mM glucose and incubated with 2.5µL of the antibody as recommended by the manufacturer for 30 min at room temperature. Then the samples were centrifuged at 214g for 5 min, washed with PBS pH 7.2/1%BSA/20mM glucose and re-suspended in 250µL of PBS for FACS. The lavage was also incubated with the relevant isotype control antibodies. The following table 2.1 summarises the antibodies by AbD Serotec

Ltd., Kidlington, UK unless otherwise mentioned. FACS plot for leukcocyte sub-populations is demonstrated in figure 2.3 and in figure 2.4 the staining for GR-1 (PMNs) and F4/80 (macrophages) during the first 24 hours of zymosan induced murine peritonitis is summarised.

Table 2.1 Antibodies used for FACS analysis of murine peritoneal leukocyte exudate

Cell Type	Antibody	Isotype
Polymorphs	Rat anti-Mouse Ly-6G & Ly-6C (Gr-1) Monoclonal antibody-PE (BD Biosciences, UK)	IgG2b
Macrophages	Rat anti Mouse F4/80 FITC (Caltag MedSystems Towcester, UK)	IgG2A
T Lymphocytes	Rat anti Mouse CD3 FITC	IgG2A
B Lymphocytes	Rat anti Mouse CD45 FITC	IgG2A
CD4+ lymphocytes	Rat anti Mouse CD4 FITC	IgG2A
CD8+ lymphocytes	Rat anti Mouse CD8 Alpha FITC	IgG2A

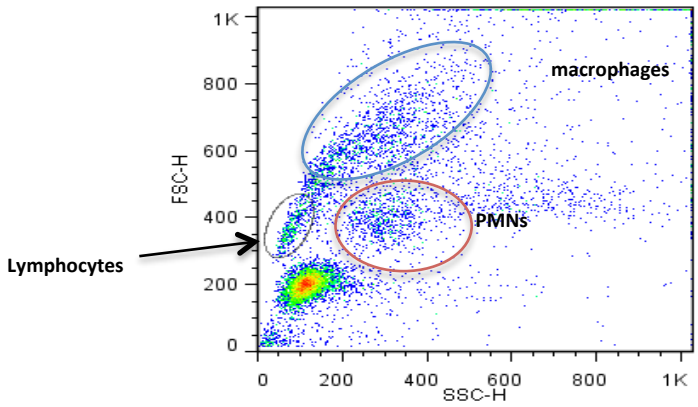


Figure 2.3 FACS plots for different leukocyte sub-populations

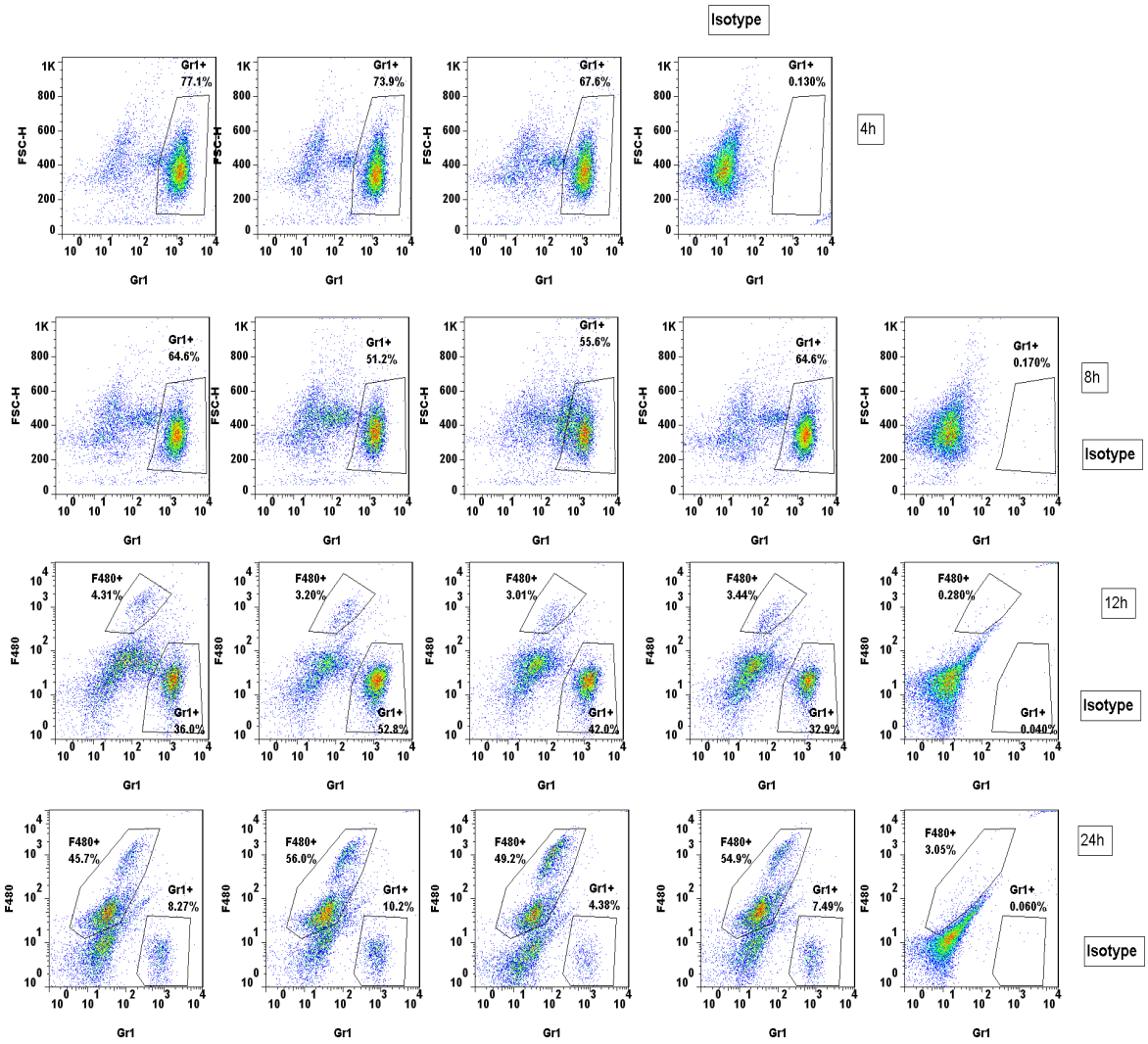


Figure 2.4 GR-1 and F/80 profile in the first 24 hours following zymosan Upper two panels are the GR-1 positive cell profile mice with the final plot of the isotype control at 4 and 8 hours following zymosan peritonitis. The lower two panels demonstrate dual staining with GR-1 and F4/80 together with isotype control (5th plot) with reduction in GR-1 numbers and rise in F4/80 positive cells as resolution ensues at 12-24 hours following zymosan.

2.4.2 Leukocyte separation

2.4.2.1 Isolation of lymphocytes from peritoneal lavage

At selected time points the mouse peritoneal cavity was lavaged as described above, cell numbers counted and then cells were centrifuged and resuspended in RPMI 1640 (RPMI) with 2% foetal calf serum (FCS) at a concentration of 1 x 10^6 cells/mL. The macrophages were separated from remaining lymphocytes by adherence to the bottom of 6-well tissue culture plates following incubation in a humidified incubator with 5% CO_2 for 90 min. Unbound cells were removed using a sterile pipette and cells numbers were estimated. The cell rich medium was then centrifuged at 484g for 7 min and suspended in the RPMI/ 2% FCS medium at a concentration of 2.5 x 10^6 cells/mL. T and B-lymphocytes were isolated using the Dynal mouse B cell and T cell negative isolation kit according to the manufacturer's instructions (Dynal Biotech Ltd).

2.4.2.2 Negative isolation of T cells

A mixture of rat monoclonal antibodies with specificity to all mouse non-T cells cells was added to cell suspensions. The antibody mix contains a mixture of rat monoclonal antibodies against mouse CD45R (B220), CD11b (Mac1), Ter-119 and CD16/32. Cells coated with the added monoclonal antibodies were then removed with magnetic beads coated with sheep polyclonal antibody to rat Ig. The cells suspension is incubated at 4°C with tilting and rotation for 45 min. Then the test tube is placed on a cold dynal magnet for 3 min and supernatant is pipetted out. This is centrifuged at 484g and re-suspended in RPMI/FCS and centrifuged again to remove any residual beads. The T cells are then resuspended in RPMI/2% FCS. Purity of the cells were regularly >95%

2.4.2.3 Negative isolation of B cells

As mentioned supernatant from the initial separation phase contained both B cells and T cells. The latter were removed by the use of a pan T-cell magnetic beads. The supernatant was first centrifuged 484g for 7 min and suspended in the RPMI/FCS medium at a concentration of 2.5 x 10^6 cells/mL and then 10 μL of the pan T cell beads were added per 10^6 target cells. The cell suspension is incubated and placed on a cold dynal magnet as described in section 2.4.2.2. The B cells are then resuspended in RPMI/FCS at the desired cell density.

2.4.3 Adoptive transfer of lymphocytes into gp91^{phox} knockout mice

Contents of resolving phase peritoneal cavities of wild type animals were isolated and macrophages separated from remaining lymphocytes by adherence to the bottom of 6-well tissue culture plates. Non-adherent cells were removed and used to isolate T and B cells as well as NK and gamma/delta T cells for adoptive transfer to gp91^{phox} knockouts using FACS and relevant antibodies to confirm that their composition and ratios reflects that present *in situ* at resolution. Resolving phase lymphocytes were enriched at a concentration of 1.0x10^6/mL and 0.5 mL injected in to gp91^{phox} knockouts. In addition, one of the problems with identifying discrete populations of cells such as that found at sites of inflammation by FACS from a larger mixed cell population is that the fluorescence of one cell type after labelling with a fluorescent antibody may be masked by the natural fluorescence of others. In order to confirm the cell types identified using cell surface antigen markers, peritoneal lymphocytes and macrophages were also isolated and put back into the FACS with their forward and side scatter signatures compared against specifically labelled cells. Thus, after lymphocytes were removed from 6-well plates above, adherent cells, mainly macrophages, were eluted with Versene, washed with 2% FCS in HBSS and re-suspended in DMEM. These cells were further depleted of contaminant T and B cells using magnetic beads coated with rat monoclonal antibodies to mouse CD3 or B220 (Dynal Biotech Ltd; Paisley, UK). T and B lymphocytes were isolated using the Dynal mouse B cell (or

T cell) negative isolation kit according to the manufacturer's instructions (Dynal Biotech Ltd) as described above.

2.4.4 Macrophage isolation and culture

2.4.4.1 Isolation of macrophages from peripheral blood sample

Peripheral venous blood samples were collected from both healthy volunteers and patients with CGD, into heparin containing syringes (5U/mL). Mononuclear cells were isolated by differential centrifugation (860g, 30 min, 20^{o}C) over Lymphoprep® (Axis-Shield, Oslo, Norway) and washed twice with sterile phosphate-buffered saline (PBS) (GIBCO, Paisley, UK) at 310g (5 min, 20^{o}C). Cells were re-suspended in 10 mL RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 100 U/mL of penicillin (GIBCO) and 100 μg/mL streptomycin (GIBCO) and 20 mM HEPES buffer and plated at a density of approximately 5x10^{6} cells/mL in 8cm^{2} Nunclon™ Surface tissue culture dishes (Fisher Scientific, Loughborough, UK). After an initial culture period of 2 h in a humidified environment at 37^{o}C, 5% CO_2, the non-adherent cells were discarded and 10 mL of fresh RPMI supplemented with 10% foetal bovine serum (10% FBS/RPMI) added to each tissue culture dish. Cells were then cultured for 5 days at in a humidified enviroment at 37^{o}C, 5% CO_2, with the addition of a further 10 mL fresh 10% FBS/RPMI after 24 h. Adherent cells were were lifted with a plastic cell lifter on day 5 and re-plated in 96-well culture plates at equal densities (10^{5}/well) in X-Vivo-15 medium (Cambrex Bioscience, Wokingham, UK). These primary monocyte-derived macrophages were incubated overnight at 37^{o}C, 5% CO_2 to adhere, and then stimulated for 24 h with 200 ng/mL Lipopolysaccharide (LPS) (Alexis, Enzo Life Science UK Ltd). These studies were approved by the Joint UCL/UCLH Committees on the Ethics of Human Research (02/0324). Written informed consent was obtained from all volunteers. No patient was studied more than once in each of the different sets of experiments.

2.4.4.2 Culture of bone marrow derived macrophages

Macrophages were obtained from the distal limb bone marrow of male mice that were 4-6 weeks old. Once the mice were sacrificed, femur and tibia were freed from all flesh and then placed in RPMI1640 with 10% FCS. In a tissue culture hood the bones were placed on a petri dish and washed for 1 minute in 70% ethanol and then transferred to RPMI/10% FCS. With the use of a 10 mL syringe the bone marrow is removed into the media and then transferred to a 50 mL tube and centrifuged at 215g for 5 min. The pellet is then re-suspended in conditioned medium which is RPMI1640, 10% FCS, 10 mM HEPES, and 15% L-conditioned media (L929 supernatant-the source of macrophage colony stimulating factor, M-CSF). The cell suspension is plated onto 10 mL per 10 cm diameter plate and incubated at humidified 37^{o}C, 5% CO_2. At day 3 further fresh RPMI/10% FCS and on day 7, RPMI/10% FCS/10 mM HEPES/15% L-conditioned media is added. On the day before stimulation media was removed and cells made less adherent with 10 mM EDTA and 4 mg/mL Lignocaine-HCL for 10 min prior to harvesting. The cell suspension is then aspirated and centrifuged at 215g for 5 min. Cells are then suspended in RPMI 1640/10% FCS and counted.

2.4.4.2.1 L929 supernatant

In order to stimulate bone marrow derived macrophages, M-CSF is needed. The source is L929 cells, which is initially plated in a T75 flask with 25 mL DMEM/10% FCS. Once confluent, the cells are washed with PBS and 100 mL of DMEM/10%FCS are added. On day 3 the medium is collected, centrifuged at 1935g for 5 min and the supernatant is then aliquoted and stored at 20% until use.

2.4.4.3 Culture of peritoneal macrophages

Peritoneum is lavaged as before, cells stained with Turks' solution and counted and re-suspended in RPMI/10% FCS at a concentration of 1x 10^6 cells/mL. Cells are then plated onto a petri dish at incubated at humidified 37^{o}C, 5% CO_2 for 1 h. Then the media is aspirated and adherent cells (peritoneal macrophages) are washed with PBS followed by incubation with Trypsin/EDTA to remove the adherent cells. Adherent cells are aspirated with repeated pipetting and with the syringe. Cells are counted; centrifuged at 215g for 5 min and re-suspended at 1x 10^6 cells/mL in RPMI/10% FCS.

2.4.5 Stimulation of leukocytes

B lymphocytes as well as macrophages (BM derived and peritoneal) from hPGD2S$^{-/-}$ and C57 black 6 wild type mice were incubated with the selective DP1 agonist (BWC245, Cayman chemicals distributed by Cambridge Bioscience Ltd, Cambridge, UK) DP_2 agonist (15-R- 15 Methyl PGD2, Cayman) or 15d-J_2 (Cayman) with media as control and then stimulated with 100 µg/mL Zymosan Type A, LPS (1µg/mL) or vehicle. Experiment was terminated 24 h later and supernatant stored at -80°C until further use. The protocol for T cells was similar apart from stimulation with anti-CD3 antibody.

2.4.6 Macrophage clearance assays

The macrophage specific stain, PKH26-PCL (Sigma, 2 mL of 500 nM) was injected into the inflamed peritoneal cavity during the resolution phase (48 h). The PKH26-PCL dye is diluted at a ratio of 1:10 in absolute ethanol and 100 µL of the diluted dye is added to 10 mL of the diluent provided with the kit. The resulting solution is mixed vigorously on vortex mixer for 15 min and then allowed to stand for further 15 min. 1 mL of the diluted dye solution is then injected to the mice intra-peritoneally. At 72 h the peritoneal cavity was lavaged, cells numbers counted and re-

suspended at $1x10^6$ cells/mL in PBS and examined by flow cytometry. Cells staining positive for PKH26-PCL (FL2 channel) were identified with FITC-labelled F4/80 (FL1 channel). Staining was found almost exclusively within macrophages (Figure 2.5). At the same time points as the peritoneal lavage, parathymic lymph nodes isolated, extracted and snap frozen in OCT1. Serial sections (minimum 15 sections per node) examined for the presence of fluorescent PKH26-PCL labelled macrophages. Two independent observers scored all sections blindly based on a previously validated analogue scale using an Olympus Axioscop microscope.

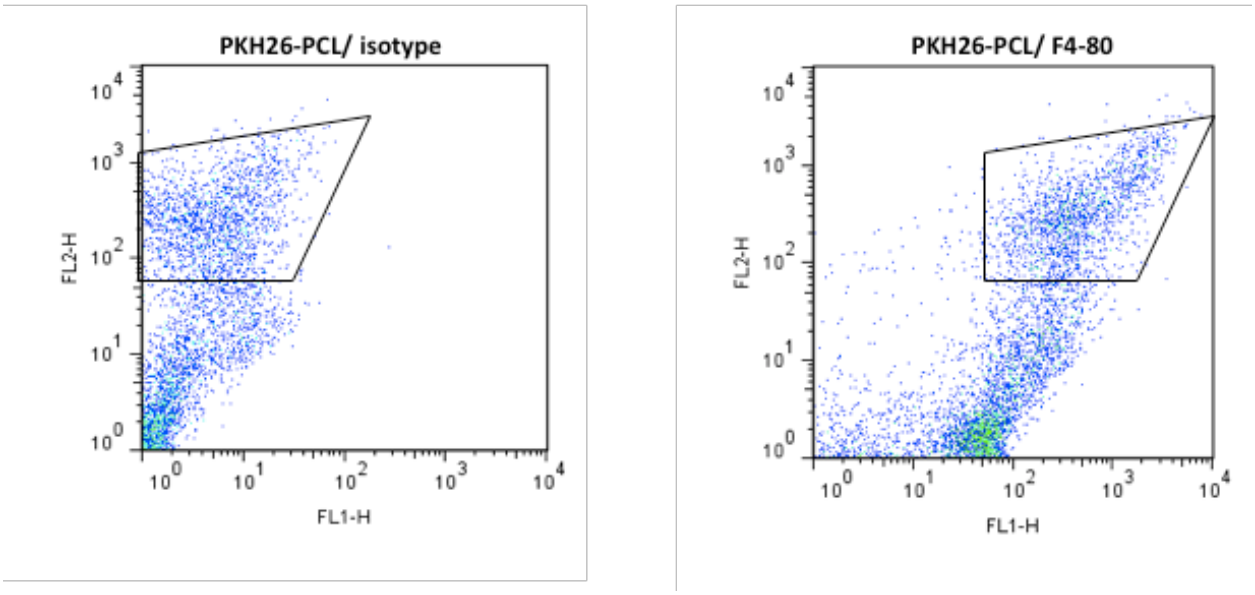


Figure 2.5 Dual labelling for PKH26-PCL and F4/80. When the leukocytes from peritoneal lavage 48 hours following zymosan were stained with PKH26-PCL (FL-2 channel), were F4/80 positive macrophages (FL-1 channel)

2.5 Measurement of apoptosis

Apoptosis was measured in the peritoneal cell rich effluents using two methods, Annexin V and Propidium iodide (PI) staining of leukocytes assessed by flow cytometry as well as Caspase-3 activity.

2.5.1 Flow Cytometry

Annexin V is a 35 kDa calcium dependent protein with high affinity for phophatidylserine [PS], which in apoptosis is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V is a sensitive marker of apoptosis as PS translocation occurs early in the apoptotic process. It is conjugated with fluorescein isothiocyanate [FITC] for identification by flow cytometry. As apoptosis leads to cell death, they eventually lose their cell wall integrity and become propidium iodide (PI) positive. By dual labelling apoptotic cells with Annexin V-FITC and PI the percentage of apoptotic cells are analysed by flow cytometry. Briefly, at selected time points 500,000 inflammatory leukocytes of the peritoneal lavage was suspended in binding buffer [10mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5mM $CaCl_2$] were incubated with 2.5 µL annexin V- FITC [BD Biosciences] and 5µL PI [BD Biosciences] for 10 min at room temperature in the dark. The samples were then using FACSCalibur and CellQuest software [BD Biosciences]. Cells staining for both annexin V and PI were defined as being apoptotic.

2.5.2 Caspase-3

The activity of caspase-3 was measured using the fluorimetric substrate Ac-DEVD-AMC. For *in vivo* experiments, frozen cell pellets were lysed in in ice-cold RIPA [20 nM Tris (pH 7.4) 150 mM NaCl, 1% NP-40, 0.5 % w/v Na deoxycholate, 0.1 % SDS (Promega)] for 10 min. After centrifugation at 16000 *g* for 15 min at 4˚C, the supernatant was aspirated and protein quantified as described below (section 2.5.2.1). The cellular protein was incubated with 50 µM substrate in

caspase assay buffer (213.5 mM HEPES, pH 7.5, 31.25% sucrose, and 0.3125% CHAPS) for 1 h, and fluorescence was measured on a microplate reader (Fluostar Galaxy, BMG Laboratory Technologies, Aylesbury, UK), with excitation at 380 nm and emission set at 460 nm. For each sample, four replicates were assayed with two replicates containing 50 μM of the caspase-3 inhibitor (Ac-DEVD-CHO), and the remaining pair containing vehicle (DMSO). Fluorescence readings from wells containing inhibitor were subtracted from total fluorescence, and results calculated as nmol AMC/ min/ per mg protein.

2.5.2.1 Quantification of protein

The protein concentrations were measured using a BCA assay (Perbio Science, Fisher Scientific, Loughborough, UK) following the manufacturer's instructions. The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colourimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid (BCA). In the first step, peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. In the second step of the colour development reaction, BCA reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance response at 562 nm with increasing protein concentrations.

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold commercially obtained lysis buffer (Mammalian Protein Extraction Buffer, GE Healthcare Life Sciences, Fisher Scientific) without protease inhibitor. Insoluble material was removed by centrifugation at 12,000 *g* for 15 min at 4^0C. Clear 96 well assay plates were used. 25 μL of cell lysates and standards were added (standard range 25-2000 μg/mL). BCA reagents A and B were

mixed together at a ratio of 50:1 before use and 200 µL was added to every well. The plate was then sealed and incubated for 30 min in an oven at 37^0C. Plates were read at 550 nm in a plate spectrophotometer (Dynex Jencons PLS Technologies Microplate Reader)

2.6 Eicasonoid analysis

2.6.1 Extraction of prostaglandins and Measurement of PGD_2

An extraction step of the cell free peritoneal exudates is required to de-proteinate the samples. One mL of the cell free peritoneal exudates is acidified with 50µL 2N HCL and kept on ice for 15 min. The SPE columns Chromabond^{TM} C-18 were primed with 4 mL 100% ethanol followed by 4 mL of distilled water at a flow rate of 5-10 mL/min using a vacuum manifold [Varian Ltd, UK]. Following this step the acidified exudate is added onto the column at a flow rate of 5 mL/min. The columns are then washed with 4 mL of distilled water followed by 15% ethanol pH 3.0. The prostaglandins are then eluted with 2 mL ethyl acetate at a flow rate of 5 mL/min, vacuum dried and stored at -80ºC.

2.6.2 Immunoassay for PGD_2

The assay is a competitive enzyme immunoassay [PGD_2-Mox EIA kit, Cayman Chemical Catalogue no. 512011]. Prior to running the assay prostaglandin D_2 is stabilized by methoximation to the PGD2-MOX derivative. This is done by heating a 1:1 solution of the sample (suspended in enzyme immunoassay buffer) and methyl oximating reagent at 60ºC for 30 min. Following methoximation the sample is added in duplicate onto the plate and incubated overnight at 4ºC. This assay is based on the competition between PGD_2-MOX and a PGD2-MOX-acetycholinesterase [AChE tracer] conjugate for a limited number of PGD_2-MOX-specific rabbit antiserum binding sites. Following incubation overnight with the AChE tracer and antiserum the plate is washed with PBS (pH 7.4) to remove any unbound reagents and then 200

μL of the Elman's reagent (which contains the substrate to AChE) is added to the well. The enzymatic reaction has a yellow colour and typically develops over 90 min in the dark. The absorbance is read at 410 nm.

2.6.3 Measurement of 15d-PGJ_2 by LC-MS-MS

Samples stored at -20^0C were thawed at room temperature and spiked with the internal standard d4-15d-PGJ_2 (4 ng) and acidified to pH 3. Solid-phase extraction was performed with 3M Varian Empore™ high performance extraction disk cartridges and the columns washed with 1 mL H_2O, 1 mL heptane and eluted with 1 mL of ethyl acetate. The eluate was dried under nitrogen and analysed by electrospray triple quadruple LC-MS-MS (Sciex API 3000, PerkinElmer, UK). The conditions for the LC-MS-MS were: C18 columns 100×0.2mm with elution volume 200 μl/min consisting of a mobile phase 0-1 minute distilled H_2O pH 3: MeCN 75:25%, followed by a gradient mobile phase to 100% MeCN. 15d-PGJ_2 was detected and quantified in negative ion mode, the electrospray potential was maintained at -4 to 4.5 kV and heated to 500°C. For MS-MS analysis, 15d-PGJ_2/internal standard were subjected to collision-induced fragmentation

2.7 Measurement of chemokines and cytokines

The chemokines MIP-1α and MCP-1 were measured by sandwich enzyme linked immunosorbent assay (ELISA). An ELISA was also used to measure IL-10, TNFα and TGFβ-1. MCP-1, IL-10 and TNFα ELISA kits were purchased by Beckton, Dickinson UK Ltd, Oxford, UK) and the MIP-1α and TGFβ-1 ELISA kits were from R&D Systems, Abingdon, UK. 96-well flat-bottom plates (Appleton Woods, Birmingham, UK) were coated with 100μl/well of diluted capture antibody in coating buffer (0.2 M sodium phosphate, pH 6.5), sealed and incubated overnight at 4^oC. Wells were aspirated and washed 3 times with 10 mM Phosphate buffered saline (PBS, 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride,

pH 7.4) and 0.05% v/v Tween-20. Non-specific binding was blocked with 200 µl/well of assay diluent (10% v/v heat inactivated fetal bovine serum in PBS pH 7.4) at room temperature for 1 h, followed by 3 washes as above. Serial dilutions in assay diluent of the MCP-1 and MIP-1α standards were added [100 µL per well] in duplicate. The samples were added in the remainder of wells and incubated for 2 h followed by 5 aspiration and washes and then incubation with the appropriate avidin-horseradish peroxidase-conjugated anti-mouse antibody for 1 h at room temperature. Wells were again aspirated and washed. The substrate tetramethylbenzidine (TMB) 100µl added to each well, plates were incubated at room temperate in the dark for $\leq$ 30 min until adequate colour development was observed. The reaction was then terminated by the addition of 50 µl 2N H_2SO_4, and the plate read at 450 nm with a subtraction at 570 nm.

2.8 Measurement of Purines

In the series of experiments looking at the role of A_2A receptor activation in inflammation and resolution, measurements of purines, adenosine, cAMP, AMP and inosine were carried out at the laboratory of Professor Edwin Jackson, Centre for Clinical Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. Proteins in cell free exudates were removed by ultrafiltration (30,000 Dalton cut-off). Purine concentrations in samples were measured by high-pressure liquid chromatography (HPLC) using ultraviolet absorption. Aliquots of each sample were injected into an Isco high-pressure liquid chromatographic system (pump model 2350, gradient programmer model 2360, V4 absorbance detector, 4.6 x 250 mm C18 column with 5 :m particle size, ChemResearch Data Management System). Mobile phase A was 0.1 M KH_2PO_4 (pH=6.1) and mobile phase B was 80 % 0.01 M KH_2PO_4 (pH=3.5) and 20% methanol. Mobile phase A was maintained at 100% for 11 min, a 2 min linear gradient to 50% mobile phase A was initiated, 50 % mobile phase A was maintained for 21 min, a 2 minute linear gradient back to 100% mobile phase A was initiated and 100% mobile phase A was maintained at least 24 min before injecting the next sample. The eluant was monitored at a wavelength of

254 nm, and the area under the chromatographic peak for cyclic AMP, AMP, adenosine and inosine were obtained. Purine levels were quantified using these areas by using reference to standard curves.

2.9 Statistical analysis

All values described in the text and figures are expressed as the mean ± standard error for *n* (number of) observations. Statistical analysis was performed using GraphPad Prism 3.01 (GraphPad Software, San Diego, USA). Statistical analysis was tailored for each experiment and detailed in each legend. Data was analysed using One way analysis of variance (ANOVA) was with Bonferroni post hoc test. Unpaired student t test was also used in chapter three to compare two groups. A p value of <0.05 was considered significant.

Chapter 3 The characterisation of inflammation in $hPGD_2S^{-/-}$ mice in response to zymosan induced peritonitis

3.1 Introduction

The innate inflammatory response that Celsus in 40 AD described as rubor (redness), calor (heat), dolor (pain), tumour (swelling) is characterised by the mobilisation of granulated polymorphonuclear (PMN) cells to neutralise the external stimuli such as invading microbes and/or trauma. This acute inflammatory response is closely followed by influx of mononuclear cells, macrophages and lymphocytes that may play a role in limiting the intensity of inflammation leading to resolution or healing(Serhan, Brain et al. 2007).

There are a diverse array of soluble lipids, vasoactive amines, cytokines and chemokines along with counter regulatory factors (glucocorticoids and adrenaline, for instance) that temper the severity of the onset phase on the one hand, and ultimately bring about its resolution on the other. Among those that drive inflammation, COX 1 and 2 are widely regarded to be pro-inflammatory by the virtue of the inhibitory actions of non steroidal anti inflammatory drugs (NSAIDs). However, work in our laboratory has shown that in a rat carrageen induced pleurisy model there are two peaks of COX-2 expression; one coincident with the acute onset phase and a second peak at resolution. The larger second peak of COX-2 is associated with the prostaglandin PGH_2 derived PGD_2 that is important for bringing about resolution (Gilroy, Colville-Nash et al. 1999).

COX metabolises phospholipase A_2-derived arachidonic acid to PGH_2, which is then converted to downstream prostanoids by a series of specific synthases. PGH_2 is converted to PGD_2 by prostaglandin D_2 synthase (Straus and Glass 2001). There are two isoforms of PGD_2 synthase, Lipocalin and haematopoietic. Lipocalin PGD_2S (L-PGD_2S) is present in the central nervous system, male genital organs as well as in the human heart. The second haematopoietic PGD_2S (hPGD_2S), a 26 kDa cytosolic protein, is present in the spleen, fallopian tubes, microvascular endothelium and hematopoietic system and being widely distributed in antigen-presenting cells,

T_h2 lymphocytes, mast cells, megakaryocytes and various macrophage cell lines where it metabolises COX-derived PGH_2 to PGD_2 (Urade and Hayaishi 2000). The latter's effect on inflammation may be either direct action on the PGD_2 receptors, DP1 and DP2 also called CRTH2 (chemoattractant receptor-homologous molecule expressed on T_h2 cells) or by its non-enzymatic metabolites, cyclopentenone PGs (cyPGs) 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and Δ^{12}-PGJ_2. They are generated from PGJ_2 by albumin-independent and -dependent reactions, respectively (Scher and Pillinger 2005).

PGD_2 acts via G-coupled receptors, DP1 and DP2 also called CRTH2 (chemoattractant receptor-homologous molecule expressed on T_h2), both of which contain seven transmembrane domains. DP1 is anti-inflammatory (Hammad, de Heer et al. 2003) and DP_2 has pro-inflammatory properties but has also been implicated in modulating allergic responses (Liu, Bleecker et al. 1990; Fujitani, Kanaoka et al. 2002; Mandal, Zhang et al. 2004). However at the point of starting my research work little was known about the precise role of PGD_2 receptors in acute inflammation.

As mentioned above, the role of PGD_2 is not limited to its action via DP1/2. Its metabolite, the cyclopentenone prostaglandin 15d-PGJ_2 is a potent activating ligand for PPARγ resulting in the inhibition of pro-inflammatory NF-κB, AP1 and signal transducers and activators of transcriptions (STATs) (Straus and Glass 2001; Scher and Pillinger 2005). It was shown to suppress TNF stimulated expression of the adhesion molecules ICAM-1 and VCAM-1 (Pasceri, Wu et al. 2000) as well as chemokines such as MCP-1. Furthermore it is a potent inducer of leukocyte apoptosis (Kawahito, Kondo et al. 2000; Ward, Dransfield et al. 2002) and directly inhibits IκB kinase that is responsible for activation of NF-κB (Rossi, Kapahi et al. 2000). However controversy exists as in whether it is present in sufficient quantities to exert immune regulatory effects (Bell-Parikh, Ide et al. 2003).

It was shown by this laboratory using a rat pleurisy model that PGH_2 derived PGD_2 and its metabolites play a role in facilitating inflammatory resolution (Gilroy, Colville-Nash et al. 1999; Gilroy, Newson et al. 2004). NSAIDs, both non-selective and COX-2 selective inhibitors block the production of all PGH_2 metabolites (PGD_2, PGE_2, Thromboxane A2 and Prostacyclin). This chapter will characterise the role of $hPGD_2S$ derived PGD_2 and15d-PGJ_2 in both acute and resolution phase of innate zymosan induced peritonitis. In order to discern this, $hPGD2S^{-/-}$ mice and wild type C57 black VI mice were used.

3.2 Materials and Methods

A detailed description of all experimental methods is described in Chapter 2. Briefly, $hPGD_2S^{-/-}$ and control wild type C57BL/6 male mice (6 weeks old) were injected with the sterile chemical stimulus Zymosan (1mg in 0.5mL PBS). Mice were sacrificed and peritoneum lavaged at 2, 6, 12, 24, 48 and 72 hours following zymosan injection. Cells were counted by haemocytometer. The differential profiles of PMNs, lymphocyte subsets and macrophages were assessed using specific fluorescent antibodies and isotype controls and then analysed using flow cytometry. The cell-free supernatants were analysed for lipid mediators and cytokines/chemokines at different time points using EIA and ELISA respectively. Furthermore, 15d-PGJ_2 was measured using LC-MS-MS. Leukocyte apoptosis was measured by both flow cytometry [with staining for Annexin V and PI] and estimation of caspase-3 activity in the cell pellets. For pharmacological rescue experiments, a selective agonist for DP1, BW245C (Narumiya and Toda 1985; Boie, Sawyer et al. 1995) and for DP2, 15(R)-15-methyl PGD_2 (Moretta, Bottino et al. 2002) were used.

Migration of macrophages from the inflamed peritoneal cavity was also examined during the resolution. In order to investigate the fate of macrophages when inflammation resolves, the fluorescent marker PKH-26L that bind to macrophages was injected 24 h following zymosan [coincident with peak macrophage numbers and onset of resolution]. During resolution of

peritonitis macrophages migrate out into draining parathymic nodes and therefore both peritoneal cells and the nodes were examined by flow cytometry and fluorescent microscopy respectively.

3.3 Results

In wild type mice, intra-peritoneal administration of zymosan resulted in a self-limiting inflammatory response that peaked at 6 h. Total cell numbers in the peritoneal effluent reached baseline levels indicant of inflammatory resolution by 48 h. This was in contrast to $hPGD_2S^{-/-}$ mice where there was an exaggerated peak inflammation compared to wild type mice at 6 hours which appeared to resolve by 24 hours. However the excess leukocyte numbers in the knockout mice at 24 h persistent during the resolution phase at 48 and 72 h suggestive of failure or delayed resolution.

3.3.1 Acute Inflammation

In the acute phase, 6 h following i.p. zymosan, there was an exaggerated inflammatory response in the $hPGD_2S^{-/-}$ mice with the total leukocyte numbers being $10.14 \pm 2.6 \times 10^6$ per mL in $hPGD_2S^{-/-}$ vs $4.7 \pm 0.94 \times 10^6$ per mL in wild type ($P<0.001$)(Figure 3.1A). Of this, Gr-1 positive granulocytes were the predominant cell type comprising $7.51 \pm 0.81 \times 10^6$ /mL in knockouts vs $2.81 \pm 1.06 \times 10^6$ per mL in WT ($P<0.001$) (Figure 3.1B). There was no significant increase in F4/80 positive macrophages at 6 h (Figure 3.1C). In $hPGD_2S^{-/-}$ mice, the increased number of PMNs could arise from either enhanced influx and/or impaired clearance from inflamed cavity by reduced programmed cell death. However $hPGD_2S$ does not influence the survival of PMNs at inflammatory sites as there was no difference in total leukocyte apoptosis between knockouts and wild types as determined by annexin (ANX) V/PI labelling (Figure 3.1D) as well as by caspase-3 activity (Figure 3.1E). This was also confirmed with regards to

apoptosis of PMNs which were gated using forward and side scatter based on Gr1 labelling on FACS (Figure 3.1 F and G).

In conclusion in $hPGD_2S^{-/-}$ mice, acute inflammation was exaggerated due to excess of PMNs but with no difference in the degree of PMN apoptosis. This finding is suggestive on increased PMN influx in $hPGD_2S^{-/-}$ mice responsible for the increased inflammation. The next step was to assess the synthesis of $hPGD_2S$ derived lipid mediator PGD_2 and its non-enzymatic cylcopentenone metabolite 15d-PGJ_2 measured in $hPGD_2S^{-/-}$ and wild type mice in this model.

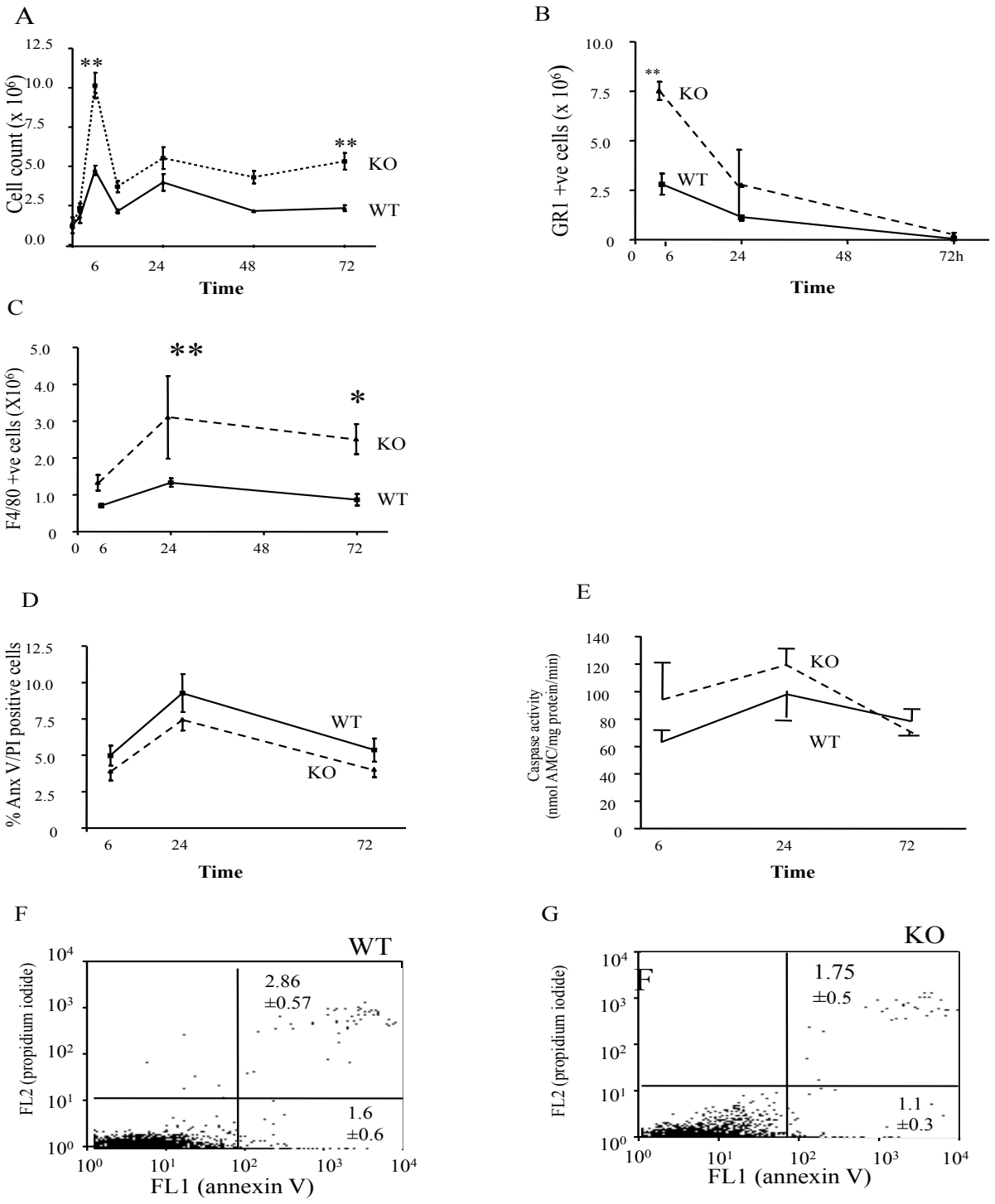


Figure 3.1 Zymosan induced peritonitis in wild type C57blackVI and hPGD$_2$S deficient mice. A- Total cell numbers in hPGD$_2$S-/- and in wild types at (P<0.001, * P<0.01). B- Excess of GR-1 positive cells in hPGD$_2$S-/- mice in the acute phase (**P<0.001). C- F4/80 positive macrophages. The numbers peak at 24 h but persist during resolution in hPGD$_2$S knockout mice (** P<0.0.01, * P<0.05). D and E- Leukocyte apoptosis when measured by annexin V/PI labelling assessed by FACS and caspase-3 measurement in the cell pellets respectively. F and G detail the FACS plots for annexin V/PI labelling for wild type mice and hPGD$_2$S deficient mice PMNs. In each group there was no statistical difference. n=6-8 animals per group. Data is represented as the mean ± SEM. *P<0.01, **P<0.001 as determined by unpaired t test.**

3.3.2 $hPGD_2S$-derived lipid mediators in acute inflammation

In the first instance levels of PGD_2 and 15d-PGJ_2 were quantified throughout the time course of resolving murine peritonitis. PGD_2 was maximal 2 h after zymosan injection with levels waning by 24 h [Figure 3.2A]. When measured by LC-MS-MS, 15d-PGJ_2 was detectable in cell-free inflammatory exudates of wild type mice, Figure 3.2B and C. It was elevated at 2 h, maximum between 6-24 h and had declined by 48/72 h with average levels of between 0.5-5ng/ml. Both PGD_2 and 15d-PGJ_2 were absent in samples from $hPGD_2S^{-/-}$ mice. Given its instability we controlled for potential *ex vivo* degradation of PGD_2 to 15d-PGJ_2 during sample processing by spiking inflammatory fluids *in situ* with deuterated PGD_2 and found that the detected 15d-PGJ_2 was native and not deuterated 15d-PGJ_2. These data, coupled with the finding that neither PGD_2 nor 15d-PGJ_2 was detectable in the exudates of $hPGD_2S$ knockouts (Figure 3.2 A and B) confirmed that 15d-PGJ_2 is a *bona fide* PGD_2 eicosanoid metabolite formed *in vivo* during resolving inflammation.

In order to understand the mechanisms behind the exaggerated acute peritonitis in the absence of PGD_2 and 15d-PGJ_2, cytokine and chemokine profiles that may play a significant role were explored.

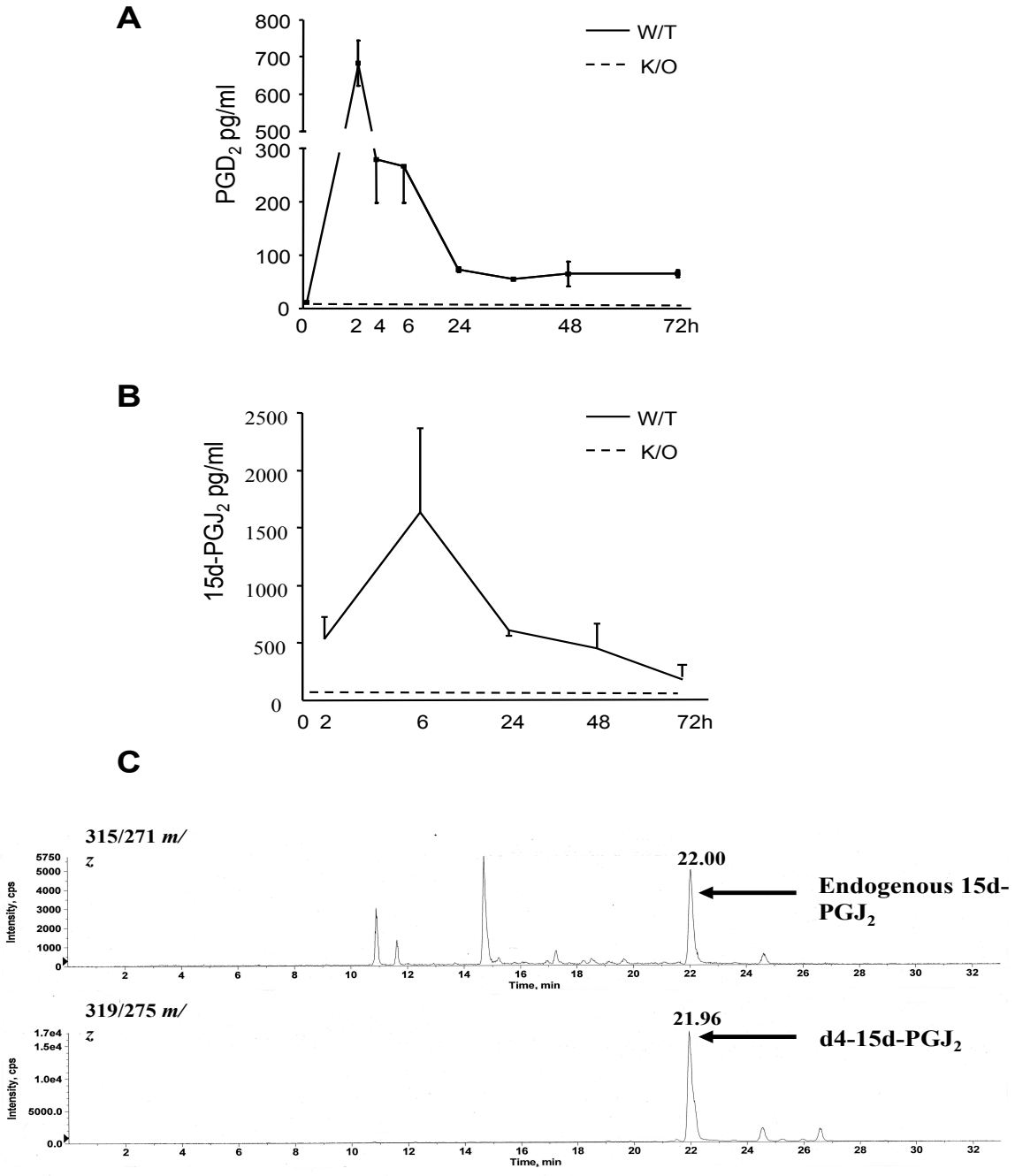


Figure 3.2 $hPGD_2S$ synthesises 15d-PGJ_2 during zymosan-induced resolving peritonitis. Figure A-PGD_2 levels in wild type C57blackV1 mice and absence in $hPGD_2S^{-/-}$ mice. B-15d-J_2 followed the peak of PGD_2 in wild type mice measured by LC-MS-MS. C- LC-MS-MS spectra – for native 15d-PGJ_2 (upper panel) and deneutrated deuterated 15d-PGJ_2 (lower panel. For eicosanoid analysis n=6-8 animals were present in each group with the exception of 15d-PGJ_2, which had 2-6 samples per group. Data is represented as the mean ± SEM.

3.3.3 Cytokine and chemokine profile in $hPGD_2S^{-/-}$ and wild type mice

The pro-inflammatory cytokine TNFα and chemokine MCP-1 as well as the anti-inflammatory cytokine IL-10 was measured at all time points following zymosan peritonitis in knockout and wild type mice.

The measured cytokines and chemokines peaked at 2 h with baseline levels by 24 h. There was a significant reduction of peak IL-10 levels (215.7 ± 53.3 pg/mL in $hPGD_2S^{-/-}$ vs. 391.7 ± 117.5 pg/mL in WT, $P<0.05$)(Figure 3.3A), whereas pro-inflammatory TNFα (362.7 ± 222.6 pg/mL in $hPGD_2S^{-/-}$ vs. 94.33 ± 76.7 pg/mL)(Figure 3.3B) and MCP-1 (10183±4094 pg/mL in $hPGD_2S^{-/-}$ vs. 3361 ± 350.2 pg/mL in WT)(Figure 3.3C) were significantly increased in $hPGD_2S^{-/-}$ mice. Therefore the exaggerated acute inflammatory state in $hPGD_2S^{-/-}$ mice at onset of inflammation could be explained, at least in part, by an imbalance between anti-inflammatory (IL-10) and pro-inflammatory (TNFα and MCP-1) mediators with decreased IL-10 and increased TNF-α and MCP-1.

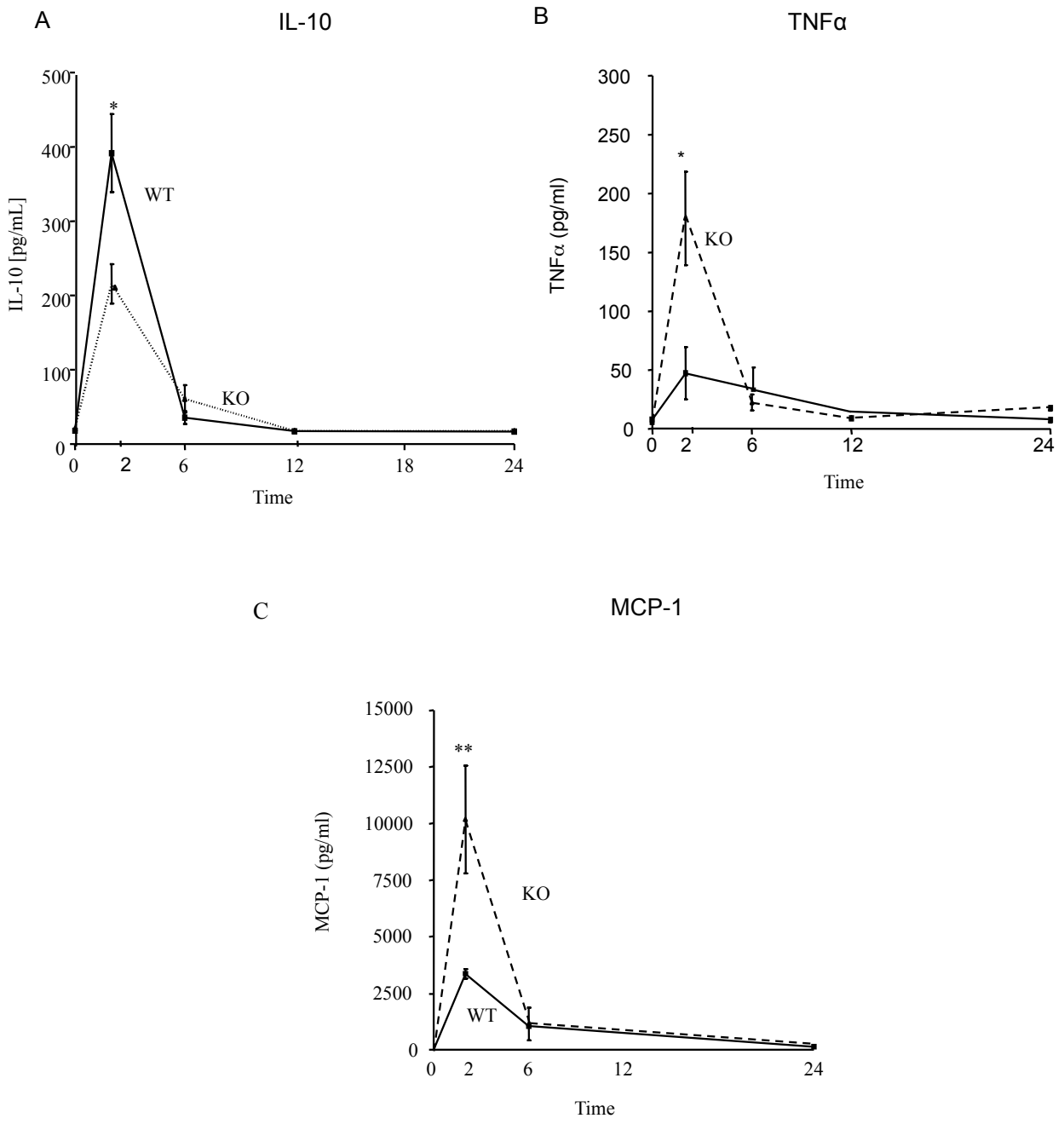


Figure 3.3 Cytokine and chemokine levels in $hPGD_2S^{-/-}$ and wild type mice in zymosan peritonitis A- IL-10 B- TNFα C- MCP-1. n= 6-8 animals per group. Data is represented as the mean ± SEM. *P<0.05, **P<0.01 as determined by unpaired t test.

3.3.4 The role of PGD_2 receptors- DP1 and DP2 in zymosan peritonitis

With respect to PMN trafficking it is not clear whether PGD_2 exerts its effect via its action on DP1 &/or DP2. To further clarify this, a selective agonist for DP1, BW245C (Narumiya and Toda 1985; Boie, Sawyer et al. 1995) and for DP2, 15(R)-15-methyl PGD_2 (Moretta, Bottino et al. 2002) were used. When $hPGD_2S^{-/-}$ mice were pre-treated with the DP1 agonist BW245C (0.03-0.3 mg/kg) 30 min prior to the i.p. Injection of zymosan, the hyper-inflammatory phenotype was rescued back to the wild type levels with reduction in total leukocyte numbers at 6 h. The greatest effect obtained with the highest dose used, 0.3mg/kg (Figure 3.4A) ($7.1 \times 10^6 \pm 1.15 \times 10^6$ /mL in BW245C treated mice vs $12.73 \pm 2.55 \times 10^6$ /mL in $hPGD_2S^{-/-}$ mice treated with vehicle). When the same experiments were done with the DP2 agonist 15(R)-15-methyl PGD_2 there was no response (Figure 3.4B). The anti-inflammatory effect of the DP1 agonist was accompanied with a rise in IL-10 [394.5±144.5 pg/mL in BW245C treated vs. 222.7±36.8 pg/mL in vehicle (Figure 3.4C) and decrease in MCP-1 (2083±1497 pg/mL in BWC treated vs. 9892±2236 pg/mL in vehicle) (Figure 3.4D) and TNFα (Figure 3.4E). There was no effect with the DP2 agonist, 15(R)-15-methyl PGD_2. The anti-inflammatory effect of BW245C was not accompanied with any changes in leukocyte apoptosis based on the observed caspase-3 activity (Figure 3.4F). The anti-inflammatory role of PGD_2 is therefore mediated via the DP1 receptor and in this model DP2 receptor does not appear to have a significant role. In conclusion, $hPGD_2S$-derived PGD_2 controls the balance between pro and anti-inflammatory cytokines/chemokines by activating the DP1 receptor.

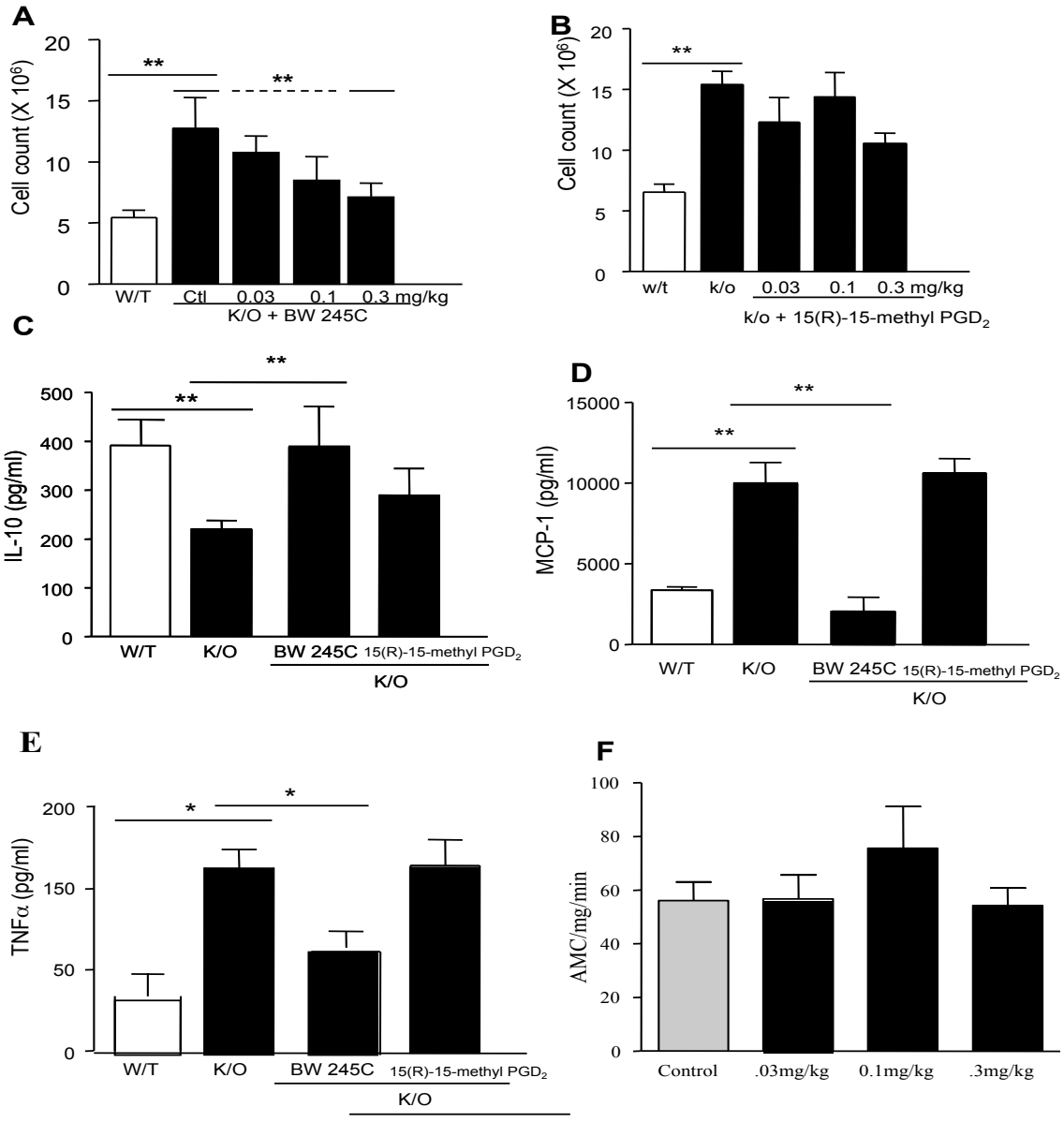


Figure 3.4 The role of DP1 and DP2 receptor agonists in acute zymosan peritonitis. Cell numbers $hPGD_2S$ knockout mice pre-treated with DP1 receptor agonist BW245C prior to zymosan injection (A), DP2 agonist 15(R)-15-methyl PGD_2 (B). When cytokines and chemokines were measured at 2 h in this model there was an increase in IL-10 (C) and a decreased in MCP-1 (D) and TNFα (E) (P<0.01, *P<0.05) with BW245c but not with 15(R)-15-methyl PGD_2. The anti-inflammatory effect of BW245C was independent of apoptosis when the leukocyte pellets were measured for caspase-3 activity (F). n= 6-8 animals per group. *, P<0.05; **, P<0.01, as determined by ANOVA, followed by bonferroni test , with data expressed as mean ± SEM.**

3.3.5 The role of 15-Deoxy-Delta-12, 14-prostaglandin J_2 [15d-PGJ_2] in acute murine peritonitis in h$PGD_2S^{-/-}$ mice

While hPGD_2S-derived PGD_2 mediates its anti-inflammatory role by activating the DP1 receptor, it is also converted, non-enzymatically, to 15d-PGJ_2. This is a potent inhibitor of NF-κB DNA and therefore has powerful pro-apoptotic and anti-inflammatory properties. As there was no difference in leukocyte apoptosis during acute inflammation in knockout and wild type mice, it is not clear whether 15d-PGJ_2 plays a role in the exaggerated peritonitis in h$PGD_2S^{-/-}$ mice. As expected, when 15d-PGJ_2 was added exogenously to WT it reduced the cell numbers in a dose dependent manner at 6 h (Figure 3.5A) by increasing leukocyte apoptosis as measured by annexin V/PI labelling (Figure 3.5B) and caspase-3 activity (Figure 3.5C). This was associated with an increase in TGFβ1 (Figure 3.5D) and Il-10 (Figure 3.5E). In the previous experiments the exaggerated acute peritonitis in h$PGD_2S^{-/-}$ mice was not due to decreased leukocyte apoptosis. Therefore 15d-PGJ_2 was administered intraperitoneally to h$PGD_2S^{-/-}$ and wild type mice at doses that do not trigger leukocyte apoptosis and there was no dose dependent reduction in cell numbers (Figure 3.5F). The absence of increased apoptosis was confirmed by little detectable annexin/PI labelling on PMNs as well as (Figure 3.5G) and little total leukocyte caspase-3 activity (Figure 3.5H).

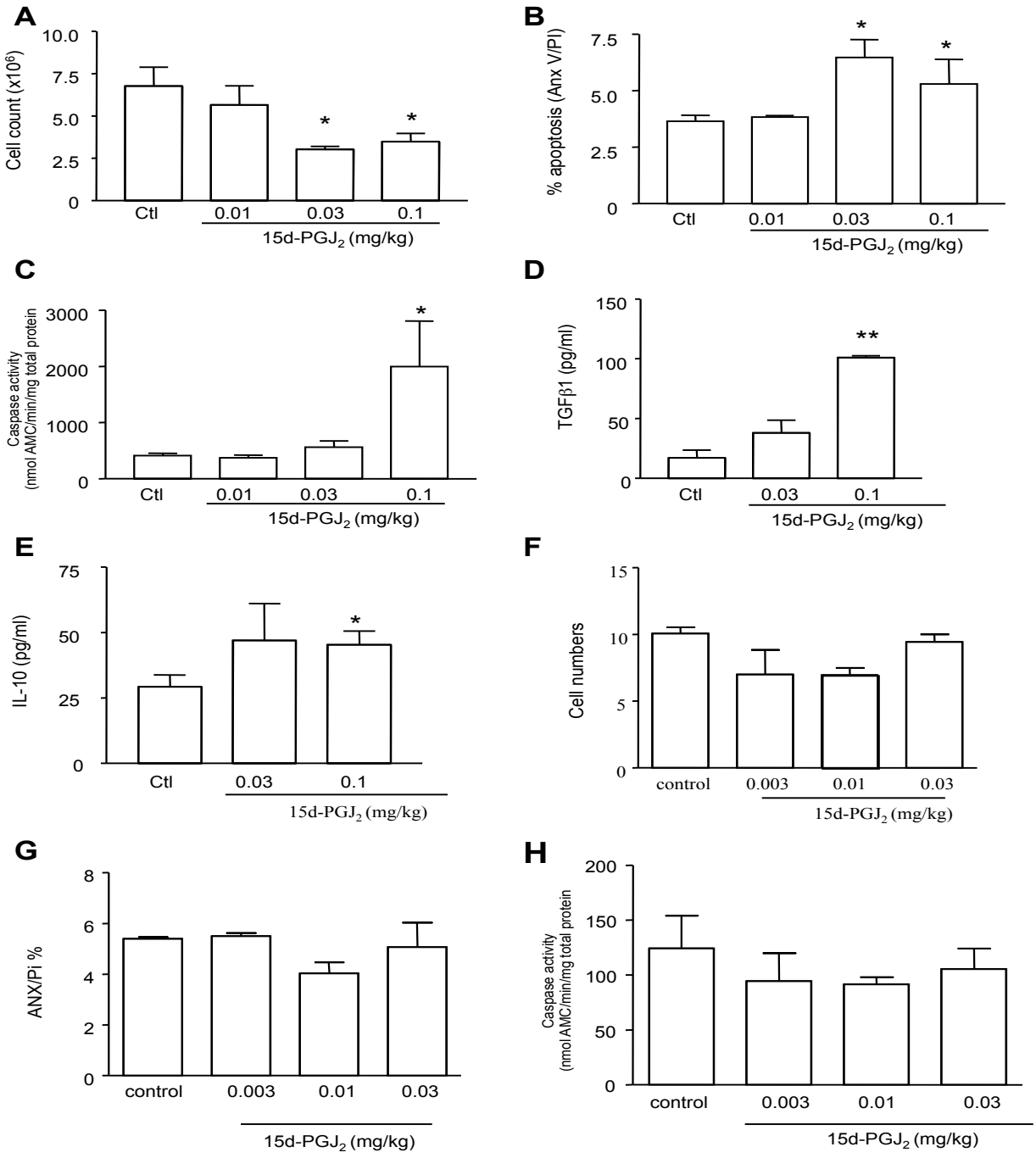


Figure 3.5 The role of 15d-PGJ_2 in zymosan peritonitis in C57blackVI wild type mice. A- dose dependent reduction in cell numbers in response to 15d-PGJ_2 at 6 h Increased leukocyte apoptosis as measured by annexinV/PI labelling (B) and by caspase-3 activity (C). This was associated with a significant increase in TGFβ (D)(P<0.01) and IL-10 (E)(*P<0.05). F- Effect on cell numbers when sub-apoptotic dose of 15d-PGJ_2 was administered to $hPGD_2S$ deficient mice. Figures G and H confirm the absence of increased apoptosis in the experiment with $hPGD_2S$ deficient mice. n= 6-8 animals per group. *, P<0.05; **, P<0.01, as determined by ANOVA, followed by bonferroni test, with data expressed as mean ± SEM.**

The above series of experiments confirm that when administered at doses that do not induce apoptosis 15d-PGJ_2 does not have anti-inflammatory properties and therefore its absence in mice $hPGD_2S^{-/-}$ may not account for the excess of leukocytes in acute peritonitis.

3.3.6 Resolution of Inflammation

As mentioned above, in $hPGD_2S^{-/-}$ mice there is a second phase of peritoneal inflammation following zymosan with an excess of total leukocyte numbers at 72 h with $5.32 \pm 1.5 \times 10^6$ per mL in $hPGD_2S^{-/-}$ vs $2.33 \pm 0.49 \times 10^6$ per mL in WT($P<0.01$) (Figure 3.1A). Whereas the exaggerated acute phase of inflammation was predominantly due to an excess of PMNs, the delayed resolution in knockout mice was due to persistence of F4/80 positive macrophages (Figure 3.1C) and accumulation of lymphocytes (Figures 3.6A-G).

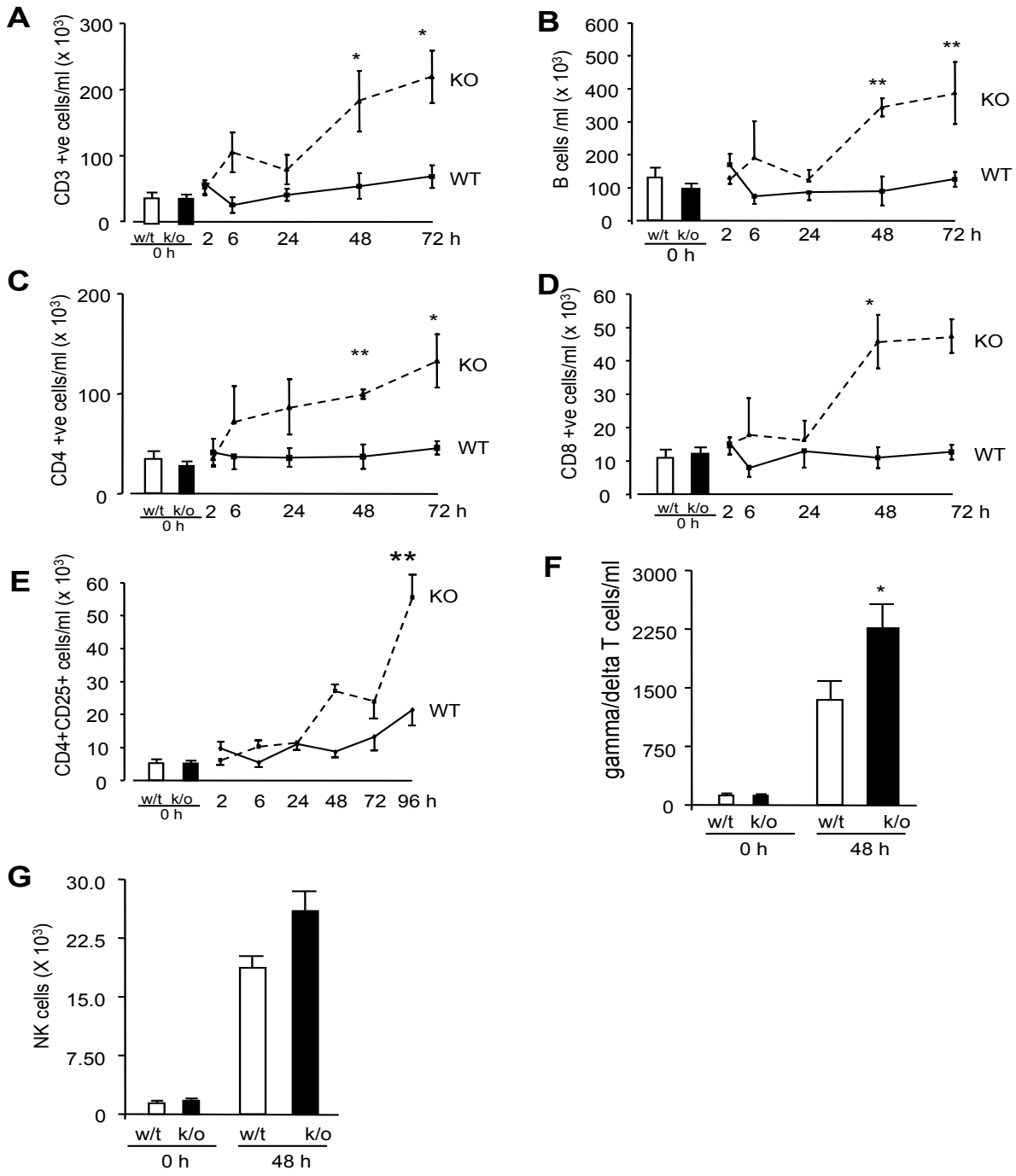


Figure 3.6 Lymphocyte profile of wild type and hPGD2S-/- mice following zymosan peritonitis by FACS. - Specific lymphocyte subtype markers was used to profile lymphocyte subsets, T cells (CD3, Figure A), B cells- CD45/Ly220 (B), CD4 (C), CD8 (D), $CD4^{+}CD25^{+}$ (E). For gamma delta T cells (F) and NK cells (G) the 48-hour time point was profiled. The appropriate iso-type was used. *, P<0.05; **, P<0.01, as determined by ANOVA, followed by bonferroni test , with data expressed as mean ± SEM.

3.3.7 Lymphocytes profile during resolution phase

The relative abundance of different subsets of lymphocytes were examined by FACS by labelling with CD3 for T cells, CD45 (B220) and CD19 for B cells. As for T cells subsets, CD4, CD8 and CD25 were examined. All subsets were significantly elevated in knockout mice at resolution phase. (Table 3.1) (Figures 3.6A-G). The persistence of lymphocytes during resolution has also been noted in carrageenan-induced paw inflammation in COX-2 deficient mice and its role is not clear (Wallace, Bak et al. 1998).

	WT mean ± SD [x10^4/mL]	hPGD$_2$S $^{-/-}$ mean ± SD [x10^4/mL]	
CD3	6.86 ± 4.49	21.92 ± 11.18	P<0.05
CD4	3.1 ± 1.77	13.29 ± 7.59	P<0.05
CD8	1.27 ± 0.58	4.74 ± 1.43	P<0.05
CD4CD25	0.89 ± 0.58	6.5 ± 6.03	P=0.05
CD45 (Ly220)	7.61 ± 5.82	38.74 ± 26.54	P<0.01

Table 3.1 Lymphocyte subsets in hPGD$_2$S $^{-/-}$ and wild type mice.

3.3.8 Failure of Macrophage efflux from the inflamed peritoneal cavity

As mentioned earlier peritonitis fails to resolve in $hPGD_2S$ deficient mice with significantly increased numbers of macrophages (Figure 3.1C) and lymphocytes (Figure 3.6). Macrophage numbers were significantly increased at 24 h in $hPGD_2S^{-/-}$ ($3.06 \pm 1.58 \times 10^6$ per mL vs. $1.33 \pm 0.19 \times 10^6$ per mL, $P<0.01$) persisting up to 72 h ($2.46 \pm 0.83 \times 10^6$ in $hPGD_2S^{-/-}$ per mL vs. $0.87 \pm 0.27 \times 10^6$ per mL in WT, $P<.0.05$) (Figure 3.1C).

The increased (though non-significant) macrophage numbers during the acute phase of inflammation in $hPGD_2S^{-/-}$ mice may have arisen from elevated MCP-1 (Figure 3.3C) and MIP1β levels (Figure 3.7A). All cytokines and chemokines measured peaked very early in acute inflammation. However, the persistence of significant numbers of macrophages in the resolution phase may be due to enhanced influx into the peritoneal cavity and/or failure to clear to the draining lymphatics. The principle clearance route for macrophage in the inflamed peritoneum as it is either local programmed cell death or clearance via the draining lymphatics to the parathymic lymph node or spleen (Bellingan, Caldwell et al. 1996; Bellingan, Xu et al. 2002). To investigate the latter, the selective macrophage label PKH26-PCL was used. When PKH26-PCL was injected i.p at the peak of macrophage accumulation (24 h), it revealed increased numbers of labelled macrophages in the peritoneal cavities of $hPGD_2S^{-/-}$ mice (Figure 3.7B) and a corresponding reduction in the parathymic node of these cells compared to wild types at 72 h (Figure 3.7C). Adding BW245C or 15d-PGJ_2 to $hPGD_2S^{-/-}$ mice reduced peritoneal macrophage accumulation (Figure 3.7D) and increased numbers of PKH26-PCL macrophages in the para-thymic lymph nodes (Figure 3.7E). The DP2 receptor agonist had no effect.

Therefore, in addition to inhibiting PMN trafficking as well as pro- and anti-inflammatory cytokine synthesis, PGD_2 also plays a role in inflammatory resolution by triggering the enhanced

efflux of macrophages from the peritoneal cavity into the draining lymphatics by the agonism of the DP1 receptor.

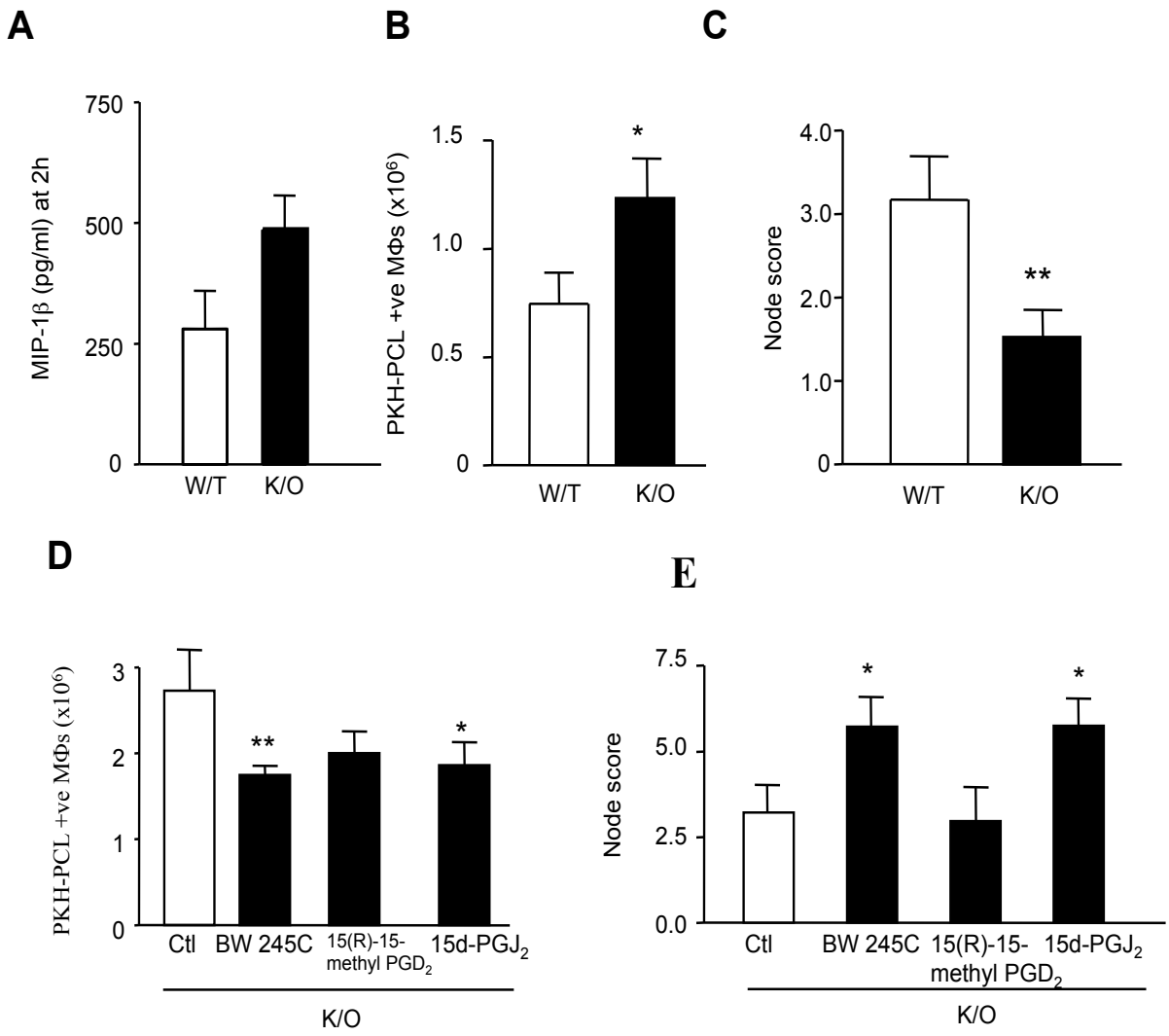


Figure 3.7 Efflux of macrophages during resolution in $hPGD_2S$-/- and wild type mice following zymosan peritonitis- In $hPGD_2S$-/- mice increased macrophage numbers could arise from enhanced accumulation following raised synthesis of MIP-1β [A] as well as MCP-1 [Figure 3.3C] and/or failure of efflux into the para-thymic nodes during resolution. To determine the latter, mice were injected i.p. with the macrophage label PKH-PCL26 at 24 and 36 h after zymosan. A significantly greater number of PKH-PCL positive macrophages were recorded in the peritoneal cavity of $hPGD_2S$-/- mice (B) compared to wild types at 72h and correspondingly fewer labelled macrophages found by histology in the para-thymic lymph nodes of the knockouts [C]. Adding BW245C (DP1 receptor agonist) or 15d-PGJ_2 to the inflamed cavity of $hPGD_2S$-/- mice reversed the accumulation of macrophages in the peritoneum (D) and resulted increased numbers of labelled macrophages in para-thymic lymph nodes [E]. n = 10 animals per group; * P≤ 0.05; **P≤0.01 as determined by ANOVA followed by Bonferoni test with data expressed as mean ± SEM.

3.3.9 Effect of hPGD$_2$S on resident macrophages and lymphocytes ex vivo

In the peritonitis model, 15d-PGJ$_2$ was not anti-inflammatory when it was administered at doses that do not trigger leukocyte apoptosis. However it is likely that hPGD$_2$S derived lipid mediators PGD$_2$ and 15d-PGJ$_2$ may have differential effects on the sub-populations of leukocytes. Thus, to garner some insight in to how PGJ$_2$ exerts its biological effects on peritoneal leukocytes *ex vivo* was examined.

In the naïve (non-inflamed) peritoneum T and B lymphocytes constitute about 40% of the total cell population (~ 1.0x10^6) with the remaining being resident macrophages which was determined using CD3, B220 and F4/80 labelling quantified by FACS. Firstly peritoneal leukocytes were lavaged followed by separation of T and B-lymphocytes as well as macrophages as described in 2.4.2.2, 2.4.2.3 and 2.4.4.3 respectively. The peritoneal leukocytes were stimulated with either LPS or zymosan (B cells and macrophages) or anti CD3 antibody (T cells). Cytokines were measured 24 h later in response to BW245C (DP1 receptor agonist), 15(R)-15-methyl PGD$_2$ (DP2 receptor agonist) or 15d-PGJ$_2$.

When stimulated with anti-CD3, IL-10 from hPGD$_2$S-deficient peritoneal T cells was significantly lower than wild types but was reversed by BW245C (DP1 agonist, 1.0 μM); neither 15(R)-15-methyl PGD$_2$ (DP2 receptor agonist, 2 μM) nor 15d-PGJ$_2$ (1 μM) had any effect, Figure 3.8A. Similarly, IL-10 secretion from zymosan or LPS-stimulated hPGD$_2$S-deficient peritoneal B cells (265±20 and 178±12 pg/mL, respectively) was lower compared to wild types (387±25 [P≤0.05] and 625±35 [P≤0.01] pg/mL, respectively) and was rescued by BW245C only [figure 3.8B-C]. When TNFα was measured from zymosan or LPS-stimulated hPGD$_2$S-deficient B cells, the levels (1625±175 and 1260±152 pg/mL, respectively) was higher compared to wild types (1160±105 [P≤0.05] and 810±65 pg/mL [P≤0.05], respectively) and were reduced by BW245C and 15d-PGJ$_2$ but not with 15(R)-15-methyl PGD$_2$ (Figure 3.8D-E).

In vitro experiments were also carried out in both peritoneal and bone marrow derived macrophages. The results were similar for both types of macrophages and with both LPS and zymosan stimulation. In contrast to T and B-lymphocytes, IL-10 from stimulated peritoneal macrophages was not elevated by BW245C (Figure 3.8F). Similarly there was no effect either with DP2 receptor activation or by 15d-PGJ_2. Also there was a differential effect of the pro-inflammatory cyto/chemokine expression of macrophages with the DP1 agonist and 15d-PGJ_2 with BW245C showing a dose dependent reduction in TNFα with zymosan and LPS (Figure 3.8G). However, when the effect on MCP-1 was examined only 15d-PGJ_2 had an effect with zymosan (Figure 3.8H).

These data show that hPGD_2S-derived lipid mediators exert differentially protective effects on peritoneal-resident (and bone marrow derived) macrophages as well as lymphocytes thereby controlling the balance of cytokines and chemokines that orchestrate innate inflammatory responses.

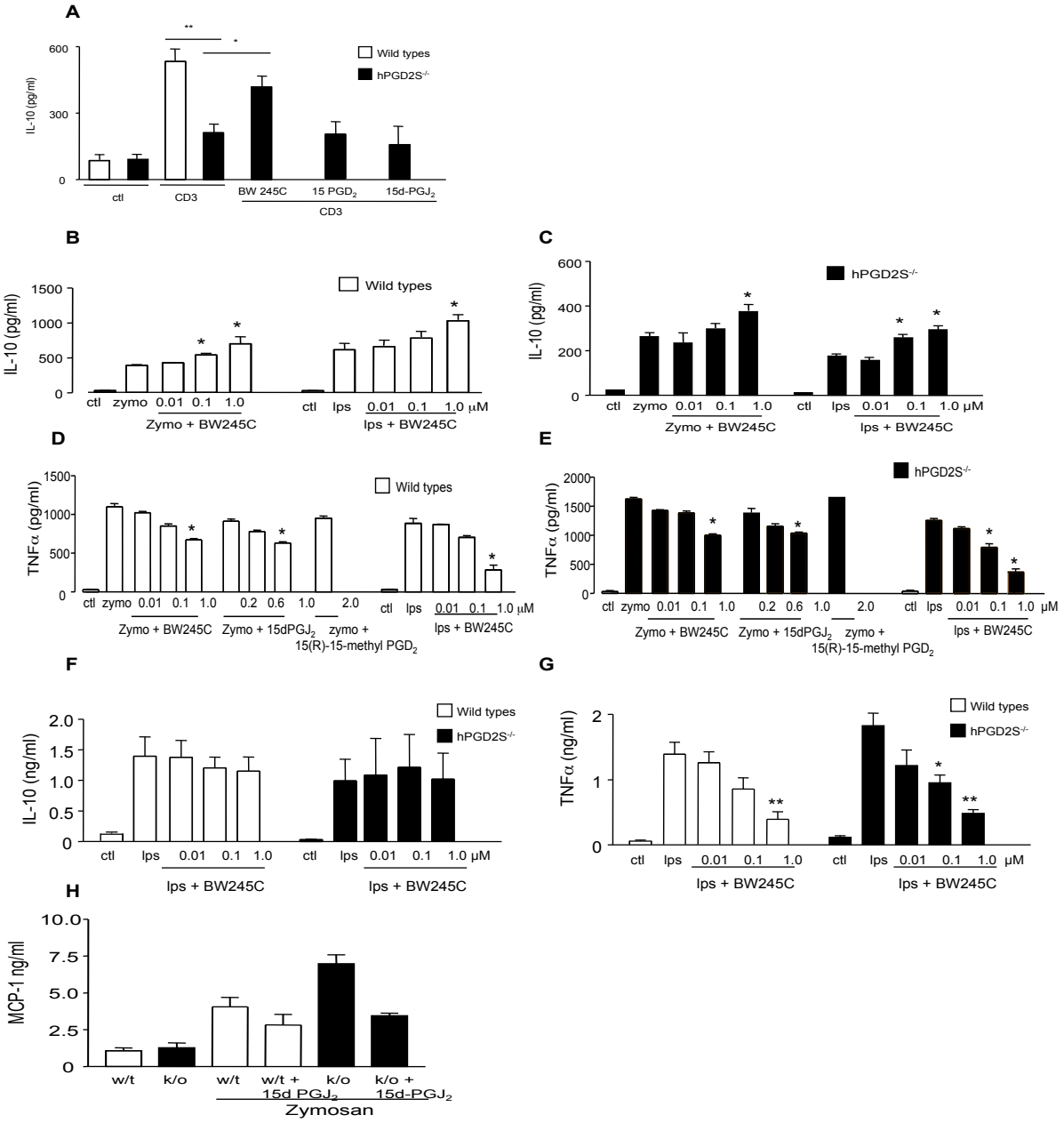


Figure 3.8 The differential effect of $hPGD_2S$ on the inflammatory phenotype of peritoneal leukocytes. IL-10 release from anti-CD3 stimulated $hPGD_2S$ deficient T cells (A) as well as LPS stimulated B cells (B and C) were not only lower than that from wild types but were rescued by DP1 activation (BW245C). DP2 receptor activation with 15(R)-15-methyl PGD_2 or 15d-PGJ_2 had no effect. Conversely, TNFα release from $hPGD_2S$ deficient B cells (D and E) was greater than that from wild types and was reversed with BW245C and 15d-PGJ_2 but not 15(R)-15-methyl PGD_2. In macrophages, whereas there was no difference in IL-10 with no effect from BW245C (F), TNFα and MCP-1 levels were higher in hPGD2S KO (G and H) with reduction in levels with BW245C (G) and 15d-PGJ2 (H) respectively. These data show the differential regulatory effect of $hPGD_2S$-derived lipid mediators on inflammatory leukocyte phenotype. Cells were isolated from n=3-10 animals and all experiments done in triplicate on two separate occasions to confirm original findings. *, P≤0.05; **, P≤0.01, as determined by ANOVA, followed by Bonferroni test, with data expressed as mean ± SEM.

3.3.10 Conclusions

A. $hPGD_2S^{-/-}$ mice have an exaggerated peritoneal inflammatory response compared to WT with zymosan

B. Enhanced inflammatory response is due to an excess of PMNs with decreased IL-10 and increased TNFα in $hPGD_2S^{-/-}$ mice

C. Excess of PMNs is not due to a difference in apoptosis between $hPGD_2S^{-/-}$ and WT mice

D. PGD_2 exerts its anti-inflammatory role by activating the G-coupled DP1 receptor and modulates the balance between anti-inflammatory IL-10 and pro-inflammatory TNFα and MCP-1.

E. 15d-PGJ_2 in sub-apoptotic dose does not exert an influence in the difference of acute peritonitis in $hPGD_2S^{-/-}$ and WT mice.

F. Resolution phase is delayed in $hPGD_2S^{-/-}$ mice and is due to an excess of macrophages and lymphocytes.

G. In $hPGD_2S^{-/-}$ mice the persistance of macrophages in zymosan peritonitis is due to enhanced influx due to increased chemokine production and impaired efflux into draining parathymic nodes. The latter is also modulated by the activation of the DP1 receptor.

H. *Ex-vivo* experiments with separated inflammatory cells suggest differential effects of PGD_2 and 15d-PGJ_2. Both activated T and B cells produce less IL-10 in $hPGD_2S^{-/-}$ reversed by the DP1 agonist whereas in activated macrophages it had no effect on IL-10 expression. TNFα production was increased in B lymphocytes from $hPGD_2S^{-/-}$ compared to WT, reversed by the DP1 agonist.

3.4 Discussion

This study demonstrates a central role for $hPGD_2S$ in controlling onset and resolution of innate immune-mediated peritoneal inflammation. Firstly, the protective nature of $hPGD_2S$ is mediated *via* DP1 and 15d-PGJ_2, which control the balance of pro- and anti-inflammatory cytokine synthesis. This is in contrast to the contradictory role of PGD_2 in allergic models of inflammation (Liu, Bleecker et al. 1990; Fujitani, Kanaoka et al. 2002; Mandal, Zhang et al. 2004). For instance PGD2 can be either pro or anti-inflammatory depending on the disease aetiology and the expression of the different eicosanoid receptor (Morris, Rajakariar et al. 2006). Signalling through DP2 (CRTH-2) causes bronchoconstriction as well as T_h2 type cells and eosinophil cell accumulation inflammation (Liu, Bleecker et al. 1990; Fujitani, Kanaoka et al. 2002; Mandal, Zhang et al. 2004). In contrast, PGD_2 activation of DP1 may also suppress asthmatic symptoms by targeting lung dendritic cells resulting in increased Treg cells that dampen inflammation in an IL-10-dependent manner (Hammad, Kool et al. 2007). As mentioned before, there is no data in the role of PGD_2 in innate inflammation. In zymosan peritonitis PGD_2 acting via the DP2 (CRTH2) receptor had no role in modulating the acute or resolution phase of inflammation. In fact more recent work done in our laboratory show that the DP2 receptor is not expressed in peritoneal inflammation. Therefore in acute inflammation both this study and work elsewhere, DP1 agonists may be an attractive and novel anti-inflammatory target capable of modulating both innate and adaptive immune responses of different aetiologies (Hammad, Kool et al. 2007).

PGD_2 is unstable *in vivo* and metabolises non-enzymatically to 15d-PGJ_2 a potent anti-inflammatory, pro-apoptotic agent (Kawahito, Kondo et al. 2000; Ward, Dransfield et al. 2002). However in $hPGD_2S^{-/-}$ mice, surprisingly, there was no difference in leukocyte apoptosis compared to wild type during acute inflammation and when 15d-PGJ_2 was administered at sub-apoptotic doses there was no effect. However *in vitro* studies show PGD_2 agonists and 15d-PGJ_2

has differential effects on leukocyte sub populations when cytokine/chemokine levels were measured (Jackson, Parhami et al. 1999; Zhang, Wang et al. 2001; Ahmed, McGettrick et al. 2011). Controversy exists over whether 15d-PGJ_2 is synthesised *in vivo* and whether it possesses relevant patho/physiological functions. This arose, in part, when earlier work in our laboratory originally reported that COX 2-derived PGD_2 and 15-dPGJ_2 brought about acute inflammatory resolution (Gilroy, Colville-Nash et al. 1999). The controversy arise because the publication suggested that COX 2 was protective at a time when the emphasis was on developing COX 2 inhibitors, but also because we used an ELISA to quantify 15d-PGJ_2. In particular, the reactive nature of 15d-PGJ_2 raised questions regarding the accuracy of using an antibody-based measuring system. This, coupled with subsequent reports using physical methods to show only negligible levels of 15d-PGJ_2 in various biological systems, collectively questioned the importance of 15d-PGJ_2 in biology (Bell-Parikh, Ide et al. 2003). However, in this study using LC-MS-MS on samples obtained from a resolving inflammation model demonstrates that 15d-PGJ_2 does exist *in vivo* at levels up to 5ng/mL. We controlled for degradation of unstable PGD_2 to 15d-PGJ_2 during sample processing by spiking inflammatory fluids *in situ* with deuterated PGD_2 and found that the detected 15d-PGJ_2 was native and not deuterated 15d-PGJ_2. These data, coupled with the finding that neither PGD_2 nor 15d-PGJ_2 was detectable in the exudates of $hPGD_2S$ knockouts (Figure 3.2A and B) and that the hyper-proliferative phenotype of $hPGD_2S^{-/-}$ T cells from a delayed type hypersensitivity reaction was reversed with 15d-PGJ_2, confirms that 15d-PGJ_2 is an endogenously-generated protective PGD_2 metabolite formed *in vivo* during resolving inflammatory reactions. The importance of this finding cannot be underestimated. cyPGs, derived from PGs of the A or D series possess anti-inflammatory (Straus and Glass 2001), anti-viral (Santoro 1997) as well as anti-cancer (Conti 2006) properties by activating either nuclear-membrane bound PPARs (Ricote, Li et al. 1998) or by forming covalent adducts with thiols *via* the unsaturated carbonyl group in the cyclopentenone moiety (Cernuda-Morollon, Pineda-Molina et al. 2001; Perez-Sala, Cernuda-Morollon et al. 2003). Importantly, protein

modification by cyPGs does not occur randomly with cyPGs targeting defined cysteine residues within certain proteins in an apparently pH dependent manner (Bickley, Ciucci et al. 2004). Moreover, structural determinants of either the protein or the cyPG may be important for the specificity of protein modification such that cyPGs with diverse structures could selectively modify distinct proteins in cells. Thus, levels of cyPGs in the extra-cellular environment may only represent the "tip-of-the-iceberg" and that their true level in biological systems may be much higher. Moreover, the lack of detectable cyPGs in biological fluids does not necessarily exclude their existence within cells and their potential to exert meaningful biological effects. Indeed, in a series of experiments we estimate that of 15d-PGJ_2 added exogenously to biological systems greater than 50% binds BSA. Moreover, in cardiomyocyte cell cultures, greater than 80% binds to culture media supplemented with 10% FCS with about 16% binding to or being metabolized by cardiomyocytes leaving less than 4% detectable by LC-MS-MS. It is for this reason that we need to add quantitatively more 15d-PGJ_2 back to inflammatory models to mimic the biological effects of endogenously produced 15d-PGJ_2 as illustrated in Figure 3.2B. We suggest that the role of 15d-PGJ_2 in self-limiting inflammatory responses is many-fold and does not necessarily overlap with that of PGD_2, controlling chemokine and cytokine synthesis as well as intracellular signalling and leukocyte apoptosis. On this theme, we found that exogenous 15d-PGJ_2 exerts pro-resolution effects if used pharmacologically by enhancing leukocyte apoptosis, the net outcome being similar to that found recently with the cyclin-dependent kinase inhibitor roscovitine, which enhanced PMN apoptosis and hastened resolution (Rossi, Sawatzky et al. 2006).

Though the control of phagocyte clearance is poorly understood, there was an excess of macrophages in the inflamed peritoneum of $hPGD_2S^{-/-}$ mice and a corresponding deficit of these cells in the draining lymph nodes compared to controls. This could have resulted from accelerated monocyte influx, mediated by elevated MCP-1 and MIP-1β (Figure 3C and Figure

7A) and/or failed lymphatic efflux. Using a phagocytosable fluorescent cell tracker, the peritoneal pool of accumulated macrophages in $hPGD_2S^{-/-}$ mice at resolution was found to be reduced by BW245C or 15d-PGJ_2 and was associated with an increase in macrophages in the parathymic lymph node. We therefore suggest that DP1 is a robust pharmacological target that, in addition to stemming PMN trafficking could be used to disseminate macrophages from sites of chronic inflammation where macrophages play a pathogenic role. A similar result was found recently with omega-3 polyunsaturated fatty-acid-derived resolvin E1 and protectin D1, which facilitated leukocyte trafficking to lymph nodes and spleen collectively underscoring the pro-resolution properties of lipid mediators in acute inflammation (Schwab, Chiang et al. 2007).

In summary, we provide proof that $hPGD_2S$ synthesises 15d-PGJ_2 during mammalian defence responses and together with PGD_2, acting through the DP1 receptor, plays a central role in controlling the onset of acute inflammation, its resolution by controlling the balance of pro- versus anti-inflammatory cytokines in addition to macrophage clearance *via* draining lymphatics. In doing so, we highlight the potentially anti-inflammatory and pro-resolution properties of cyPGs and DP1 receptors.

Chapter 4 Biphasic trafficking of lymphocytes during acute inflammation

4.1 Introduction

Inflammation is controlled by a balance of pro- and anti-inflammatory signals resulting in the development of an immune response followed by temporally released pro-resolution factors that lead to inflammation switching off and injured tissues returning to normal physiology (Nathan 2002; Gilroy, Lawrence et al. 2004; Gilroy, Feldmann et al. 2010). In chapter 3, I demonstrated a pivotal role for PGD_2 in innate inflammation both at onset and during resolution via its action on the DP1 receptor in modulating PMN trafficking and clearance of macrophages respectively(Rajakariar, Hilliard et al. 2007). Its role in lymphocyte expression and trafficking is less clear.

In the naïve mouse peritoneal cavity, lymphocytes constitute half the cell population of which approximately 70-80% are B lymphocytes. Predominant B lymphocyte population is of B-2 subset with surface expression of CD23 that contributes to overall 80- 99% of circulating B cells (von Boehmer and Melchers 2010). However another subset, B1, characterized by the surface expression of CD5 is the predominant B lymphocytes in the pleural and peritoneal cavity (Hayakawa, Hardy et al. 1983; Wortis and Berland 2001). One of the major impetuses for this current investigation stemmed from the observation of lymphocytes repopulating peritoneum as inflammation abates in zymosan peritonitis. This has also been observed previously in our laboratory (Gilroy, Colville-Nash et al. 2003) and by others (Takada, Hiromatsu et al. 1993; Wallace, Bak et al. 1998; McLoughlin, Jenkins et al. 2005; Hoffmann, Priller et al. 2007), suggesting that lymphocytes might help to switch off acute inflammation. Traditionally less weight has been placed on the role of lymphocytes in innate inflammation. Given their roles in the early defence to bacteria and viruses, innate-type lymphocytes including B1 cells merit further exploration for their potential roles in host defence and restorative physiology as its becoming clear that diminished innate lymphocyte function or enhanced lymphocyte death by apoptosis, for instance, has being postulated to play a central role in the pathogenesis of burn

injury (Ditschkowski, Kreuzfelder et al. 1999; Godshall, Scott et al. 2003) and sepsis (Hotchkiss, Tinsley et al. 1999; Hotchkiss, Chang et al. 2000), respectively.

The objective of this chapter is to characterize the lymphocyte profile in the acute and resolution of zymosan induced peritonitis and the role of PGD_2 in lymphocyte trafficking. Firstly lymphocyte profile in a human model of peritonitis will be examined. By using flow cytometry, lymphocyte subtypes will be characterized in a mouse zymosan peritonitis model and the role of PGD_2 will be investigated. Zymosan peritonitis in lymphocyte deficient $RAG1^{-/-}$ will lead to further insights in the role of repopulating lymphocytes in inflammatory resolution.

4.2 Material and Methods

Detailed experimental methods are described in chapter 2. Briefly, peritonitis was induced in mice by the i.p. injection of type A zymosan (1 mg, for all experiments unless otherwise stated), group B streptococcus (GBS) or LPS (1 mg/kg). Ethical approval [P/03/136A] was obtained for collection of human peritonitis samples from St. Bartholomew's & the Royal London Hospitals from chronic kidney disease (CKD) stage 5 patients undergoing peritoneal dialysis. To determining the fate of peritoneal T and B cells, cavities of mice bearing a 4 h zymosan-induced peritonitis was lavaged with sterile PBS to remove accumulated inflammatory cells and oedema. 5 mL of pre-warmed 5% trypsin was then added to the peritoneal cavity for 10 min followed by an equal volume of complete medium to acquire cells adhered to the peritoneal lining/greater omental lymphoid organ. Cells were then analysed by FACS. Cytokines were measured by ELISA according to manufacturer's instructions (eBiosciences, Hatfield, UK). Antibodies, CD3/CD19 (AbD Serotec, Oxford, UK), B cells (Ly220, AbD Serotec), CD5 (BD Bioscience), MAC-1/CD11b (BD Bioscience), NK and gamma/delta cells (gift from Dr. T. Hussell, Kennedy Institute, UK), GR1 (BD Bioscience, UK) or F4/80 (Caltag Medsystems, Buckingham, UK) using respective isotype antibodies as controls (AbD Serotec) were used. For apoptosis, cells

were incubated with annexin V/propidium iodide (Becton Dickinson), and run on Becton Dickinson FACScalibur with data analysed by Cellquest. For pharmacological rescue experiments, a selective agonist for DP1, BW245C (Narumiya and Toda 1985; Boie, Sawyer et al. 1995) and for DP2, 15(R)-15-methyl PGD_2 (Moretta, Bottino et al. 2002) were used.

4.2.1 Adoptive transfer of lymphocytes

Contents of resolving phase peritoneal cavities of wild type animals were isolated and macrophages separated from remaining lymphocytes by adherence to the bottom of 6-well tissue culture plates. Non-adherent cells were removed and used to isolate T and B cells as well as NK and gamma/delta T cells for adoptive transfer to $gp91^{phox}$ knockouts using FACS and relevant antibodies to confirm that their composition and ratios reflects that present *in situ* at resolution. Resolving phase lymphocytes were enriched at a concentration of $1.0x10^6$/mL and 0.5 mL injected in to $gp91^{phox}$ knockouts.

4.3 Results

4.3.1 Lymphocyte profile in human peritonitis

Peritonitis is the most common complication in CKD-5 patients on peritoneal dialysis. Clinical assessment of these patients was based on patients presenting with abdominal pain, cloudy dialysate and leucocyte count of greater than $100/mm^3$. In patients who develop peritonitis, the overnight effluent was examined for different cell types. In all cases studied, peritonitis resolved by day 5 determined by the appearance of a clear dialysate and abatement of abdominal symptoms (Figure 4.1A). At the onset of peritonitis as expected PMNs was the predominant cells type (Figure 4.1B). It became apparent that as inflammation decreases, lymphocyte numbers increase (Figure 4.1C). Therefore as observed in animal models, lymphocytes re-populate the site of injury as inflammation resolves.

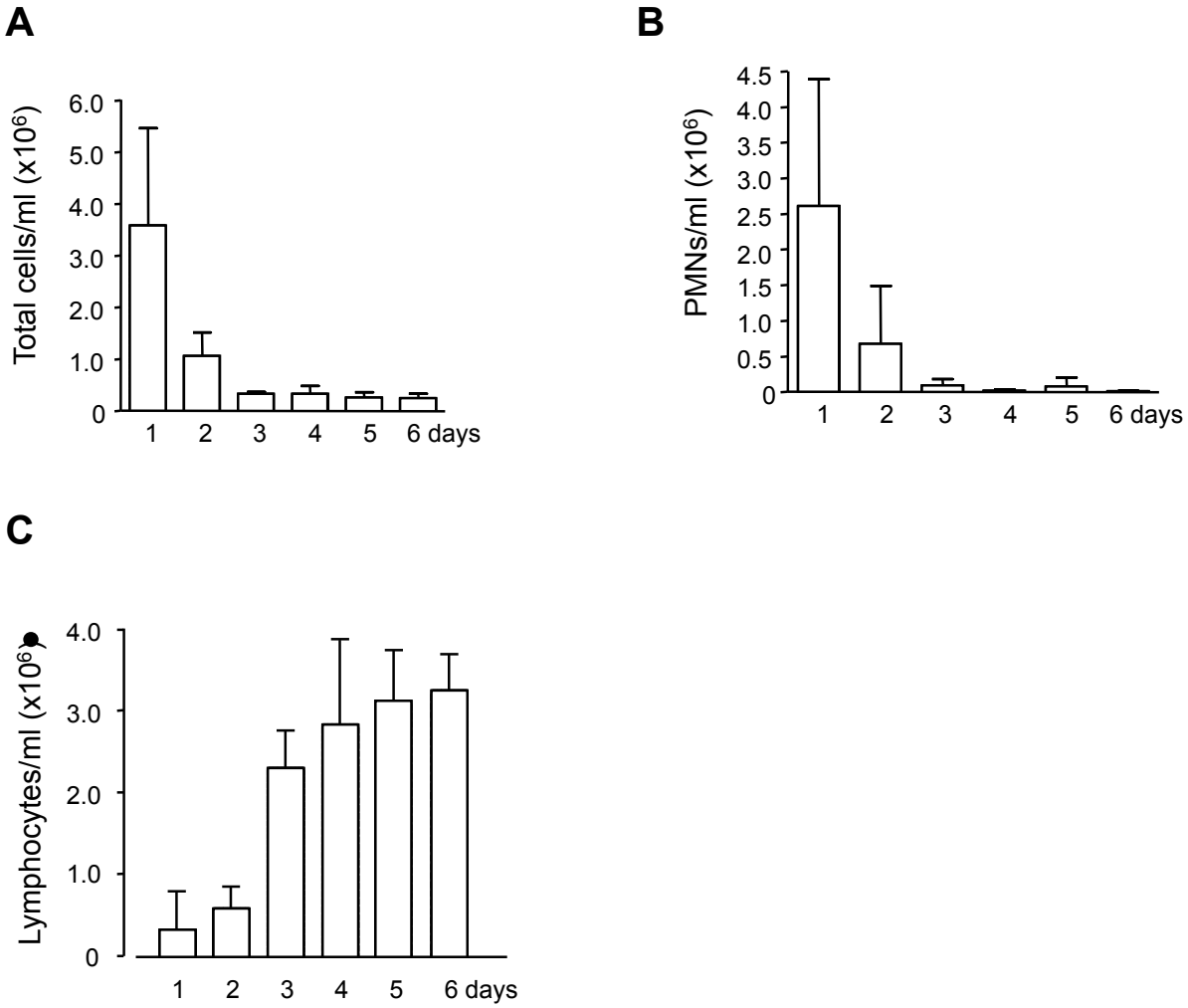


Figure 4.1 Peritoneal cellular profile in patients undergoing peritoneal dialysis who develop acute resolving peritonitis. A- Total leukocyte profile. B- The PMN and C- Total lymphocyte numbers. Data expressed as mean ± SEM.

4.3.2 Lymphocyte profile in murine zymosan peritonitis

Total cells numbers and lymphocytes were examined in a resolving zymosan murine peritonitis (Figure 4.2A), which again demonstrated a biphasic response with reduction in lymphocyte numbers in peak inflammation with repopulation during resolution. Lymphocytes constitute about 50% of the total cell population with the remaining being resident macrophages.

4.3.2.1 T Lymphocytes

In the naïve murine peritoneum (0 h) $CD4^+$ and $CD8^+$ cells were found as well as lower numbers of $CD4^+/CD25^+$, gamma/delta T cells and NK cells (Figure 4.2B-G). As lymphocytes repopulated the peritoneum, there were more $CD4^+/CD25^+$, gamma/delta T cells and NK cells found during resolution (Figure 4.2E-G).

4.3.2.2 B Lymphocytes

The majority of lymphocytes in the naïve cavity are B cells constituting about 70-80% of the total lymphocyte population labelling positively for CD19 as well as B220. Of these B cells about 80% are $B220^{low}/CD5^+/MAC\text{-}1^{low}$, indicative of a B1 phenotype with the remainder being $B220^{high}/MAC\text{-}1^-$ B2 cells (Figure 4.3A-D). As inflammation initiates (1-4 h), B cells disappear but repopulate the peritoneum again between 12-24 h (Figure 4.3E). Notably there were more B1 cells expressing higher levels of MAC-1 than in the naïve state (Figure 4.3C). As inflammation in this model peaks between 6-12h and subsequently resolves, lymphocytes appear to repopulate the peritoneum during or just after resolution. Experimentally enhancing the severity of the inflammatory response within the peritoneum by injecting three doses of zymosan (0.1, 1.0 and 10 mg) to three separate groups of mice and therefore prolonging resolution, is associated with delayed lymphocyte repopulation, Figure 4.3F. Whether this is a delay or suppression is unclear.

Thus, from the above experiments we show a shift in lymphocyte populations from the naïve to a resolving state constituting more innate-type lymphocytes as well as a different phenotype of B1 cells.

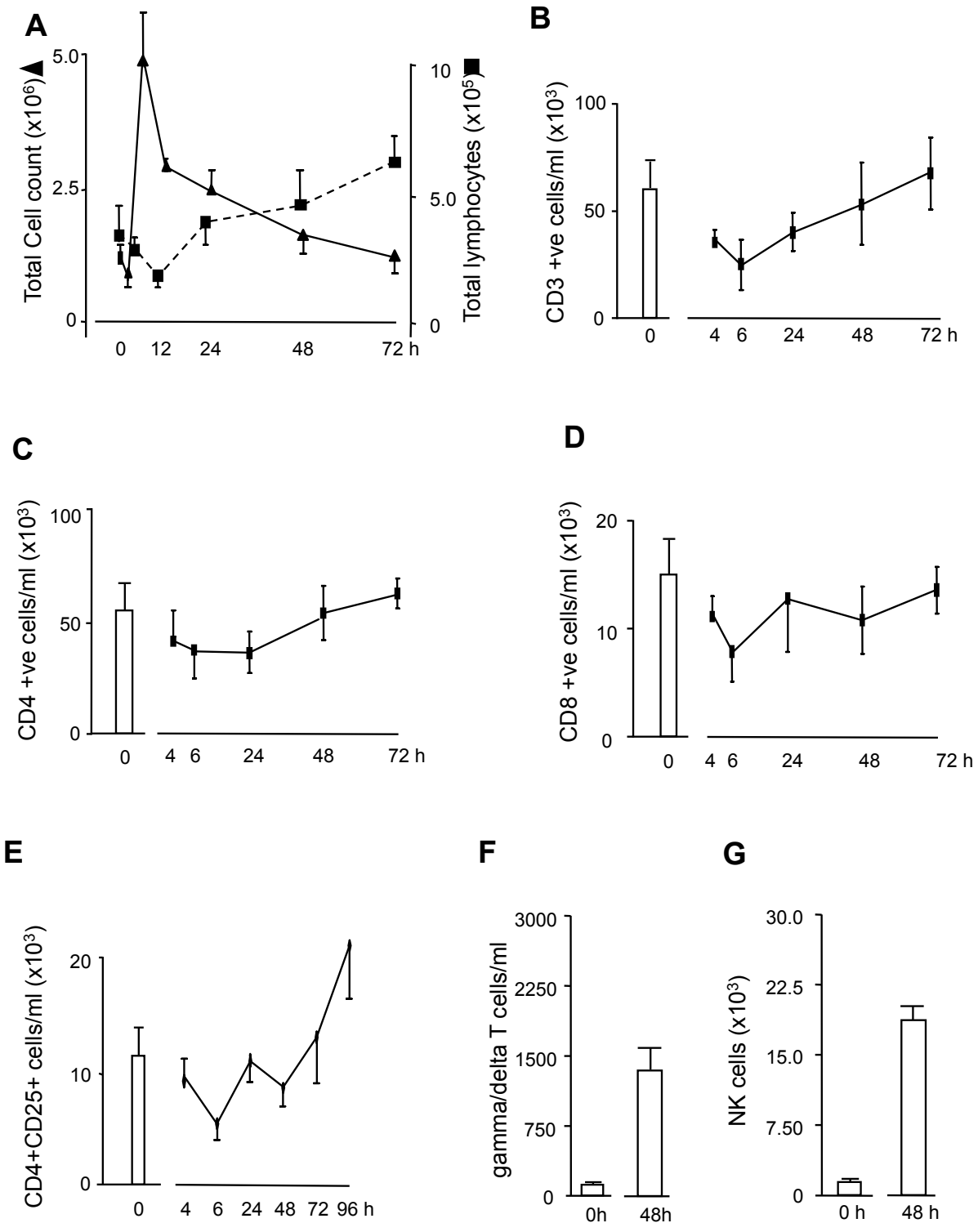


Figure 4.2 T lymphocyte profile in murine zymosan-induced peritonitis. A- During peak PMN rich inflammation, lymphocyte numbers reached a nadir and re-populate during resolution phase. B-G- The profile of T lymphocytes, CD3, CD4, CD8, $CD4^+/CD25^+$, gamma/delta T cells and NK cells. . *n* = 6–8 animals per group. Data expressed as mean ± SEM.

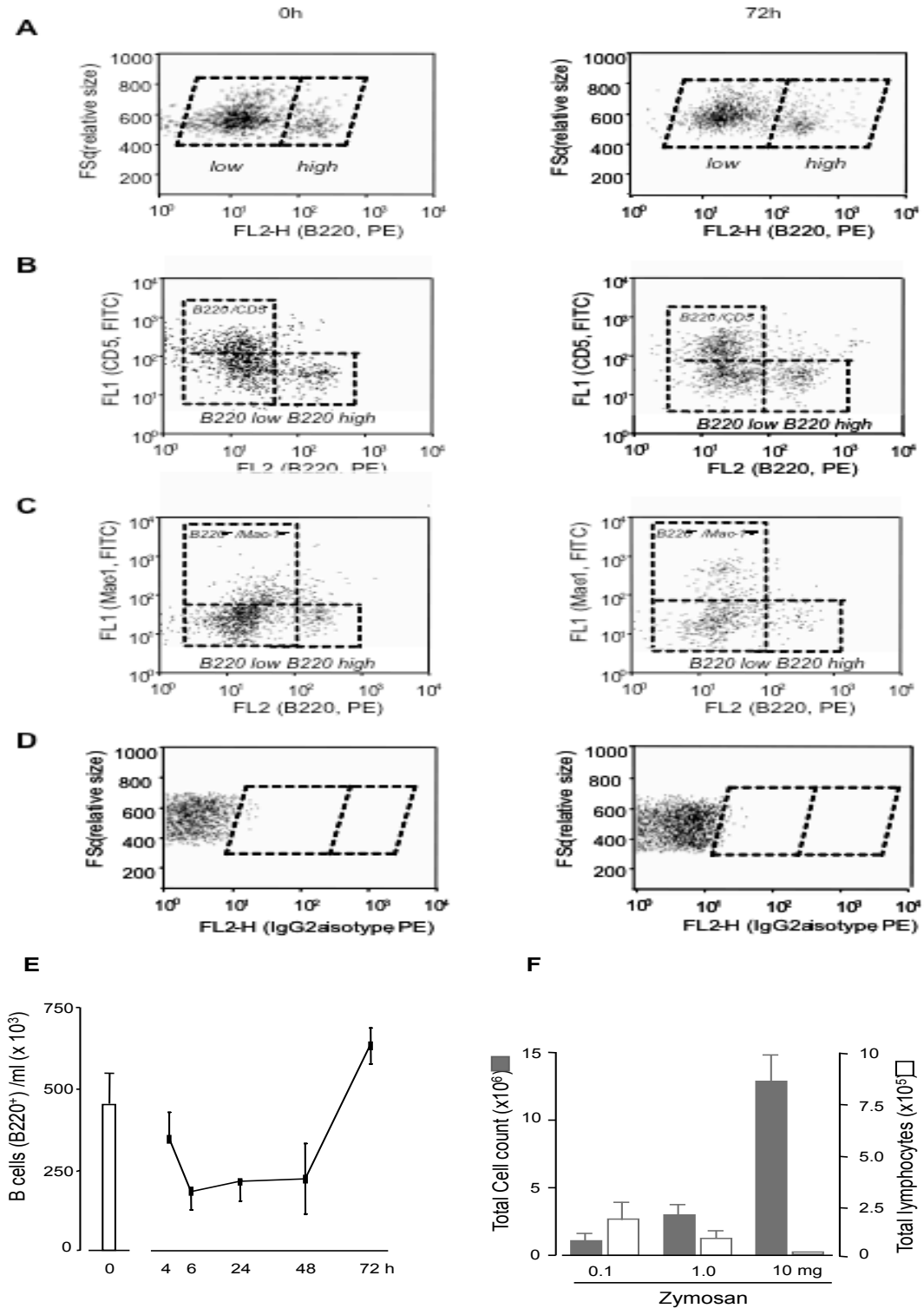


Figure 4.3 B lymphocyte profile in murine zymosan-induced peritonitis. A-D FACS plot of B cells, which constitute 80% of total lymphocyte population that label for CD19 as well as B220. Of these B cells about 80% are $B220^{low}/CD5^{+}/MAC\text{-}1^{low}$, indicative of a B1 phenotype with the remainder being $B220^{high}/MAC\text{-}1^{-}$ B2 cells. E- Total B lymphocyte profile during resolving inflammation. F- Inflammatory response within the peritoneum by injecting three separate doses of zymosan (0.1, 1.0 and 10 mg) that prolongs resolution and delays lymphocyte repopulation (72 h) *n* = 6–8 animals per group.

4.3.3 Peritoneal lymphocyte disappear in response to PGD_2

Investigating how lymphocytes disappear we recorded equivalent numbers of T and B lymphocytes in the peritoneal cavity $hPGD_2S$ knockout mice at 6h as in the naïve cavity (0h) of wild types (Figure 4.4A-E). Furthermore in the peritonitis time course, even though lymphocyte numbers reduced during the acute phase, there still was an excess in the $hPGD_2S^{-/-}$ mice. Adding BW245C (DP1 agonist) to $hPGD_2S^{-/-}$ reduced lymphocytes at 6h with the clearance effects of DP1 being on B cells, Figure 4F. 15(R)-15-methyl PGD_2 (DP2 agonist) had no effect on lymphocyte numbers in knockouts indicating that DP1 receptor activation is responsible for B cells disappearance early in acute inflammation. The fate of CD3 positive cells remains less clear. Two possibilities include adherence to the omental lymphoid organ, the so-called "leukocyte disappearance reaction" typical of peritoneal macrophages during acute peritonitis and lymphocyte apoptosis. To investigate the former, 5% trypsin was added to a 4 h inflamed cavity for 10mins recovered displaced macrophages but not lymphocytes, Figure 4G. As for apoptosis there was no difference in percentage annexin V/PI labelling between $hPGD_2S^{-/-}$ and wild type mice. Therefore, I suspect that within a few hours of inducing an inflammatory response, peritoneal resident B cells disappear in a PGD_2/DP1 dependent manner but that the fate of CD3 positive cells remains unclear.

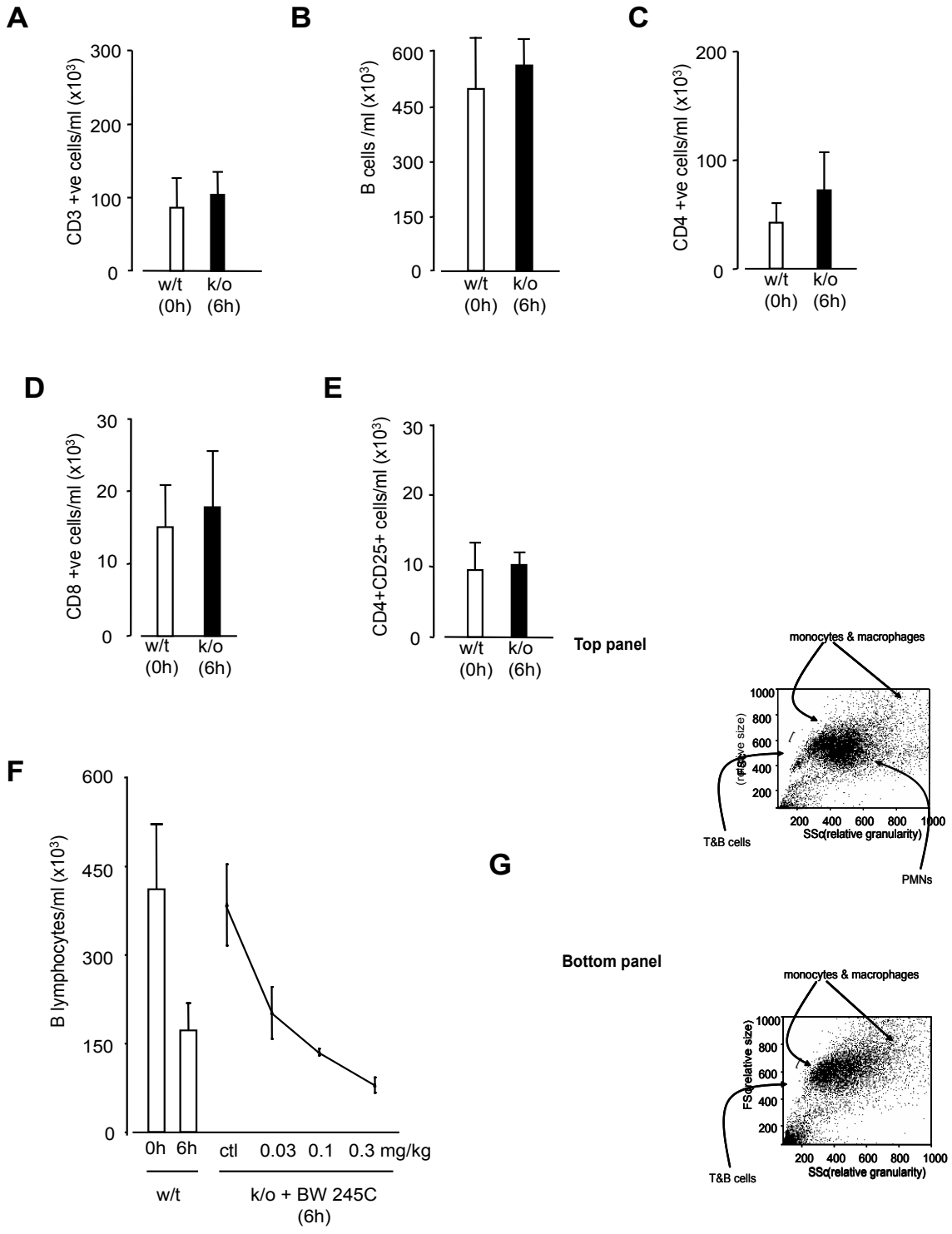


Figure 4.4 PGD_2 controls the clearance of peritoneal-resident lymphocytes. Figures A-E In response to inflammatory stimuli, lymphocytes in the peritoneum disappear between 6-24 h. However, lymphocyte numbers in $hPGD_2S$ knockouts at 6 h (filled bars) were found to be equivalent to that present in the naïve cavity of wild types (open bars) suggesting a role for either PGD_2 and/or15d-PGJ_2 in the initial clearance of lymphocytes. Figure F Adding back BW245C (DP1 receptor agonist) to $hPGD_2S$ knockouts caused a reduction in B cells. Figure G FACS analysis of leukocytes following trypsinisation of the peritoneal cavity at 4h following zymosan injection (bottom panel). *n* = 8 animals per group. Data expressed as mean ± SEM.

4.3.4 **Resolution phase lymphocytes are protective against super-infection**

To examine the role of lymphocytes in acute inflammation, zymosan was injected into the peritoneal cavity of lymphocyte-deficient RAG1$^{-/-}$ (recombination activation gene -2) mice. RAG proteins initiate the recombination process of the gene elements encoding the variable (V), diversity (D), and joining (J) segments. This leads to the generation of a diverse repertoire of antigen-specific receptors at the surface of T and B lymphocytes. RAG1$^{-/-}$ mice therefore lack T and B cells due to an early block of lymphocyte development and proliferation but have natural killer (NK) cells. In RAG1$^{-/-}$ mice inflammation at onset was greatly exaggerated, being twice that in wild types (Figure 4.5A) with the principle cell type being PMNs (Figure 4.5B). This exaggerated response in RAG1 knockouts was associated with decreased exudate IL-10 and elevated TNFα levels (Figure 4.5C). However, despite an elevated inflammatory response in knockouts, inflammation in wild types and RAG1$^{-/-}$ resolved uniformly from 24 h onwards (Figures 4.5A and B) suggesting that lymphocytes have no role in switching off acute inflammation. As demonstrated above the phenotype of lymphocytes in the peritoneum during resolution differs slightly from that in the naïve cavity. Therefore pursuing the idea that lymphocyte may protect against secondary infection, in a second experiment RAG1$^{-/-}$ and wild type mice were injected with a sub-lethal dose of GBS 48 h after zymosan injection. Thus, live bacteria were introduced into the inflamed cavity as inflammation resolved and its effects determined 24 h later (Figure 4.5D). Injecting GBS into RAG1$^{-/-}$ mice 48 h after receiving zymosan showed an approximate doubling of inflammatory cell accumulation compared with wild types treated in the same way (Figure 4.5D). This resulted in a lower bacterial load in the plasma of GBS-treated RAG1$^{-/-}$ (Figure 4.5E) but substantially accelerated mortality (Figure 4.5F). Furthermore, wild types mice that received zymosan followed by bacteria displayed fewer signs of illness compared to those that received GBS alone, which exhibited 50% mortality by 24 h with the remaining animals dying by 48 h. Results from these studies suggest that lymphocytes modulate host responses to injury/infection but are not required to bring about resolution i.e.

clear PMNs and macrophages from inflamed sites. In terms of controlling initial leukocyte trafficking in response to non-specific stimuli, protection is conferred by resident lymphocytes with resistance to secondary infection being exerted by repopulating, phenotypically different resolution-phase lymphocytes.

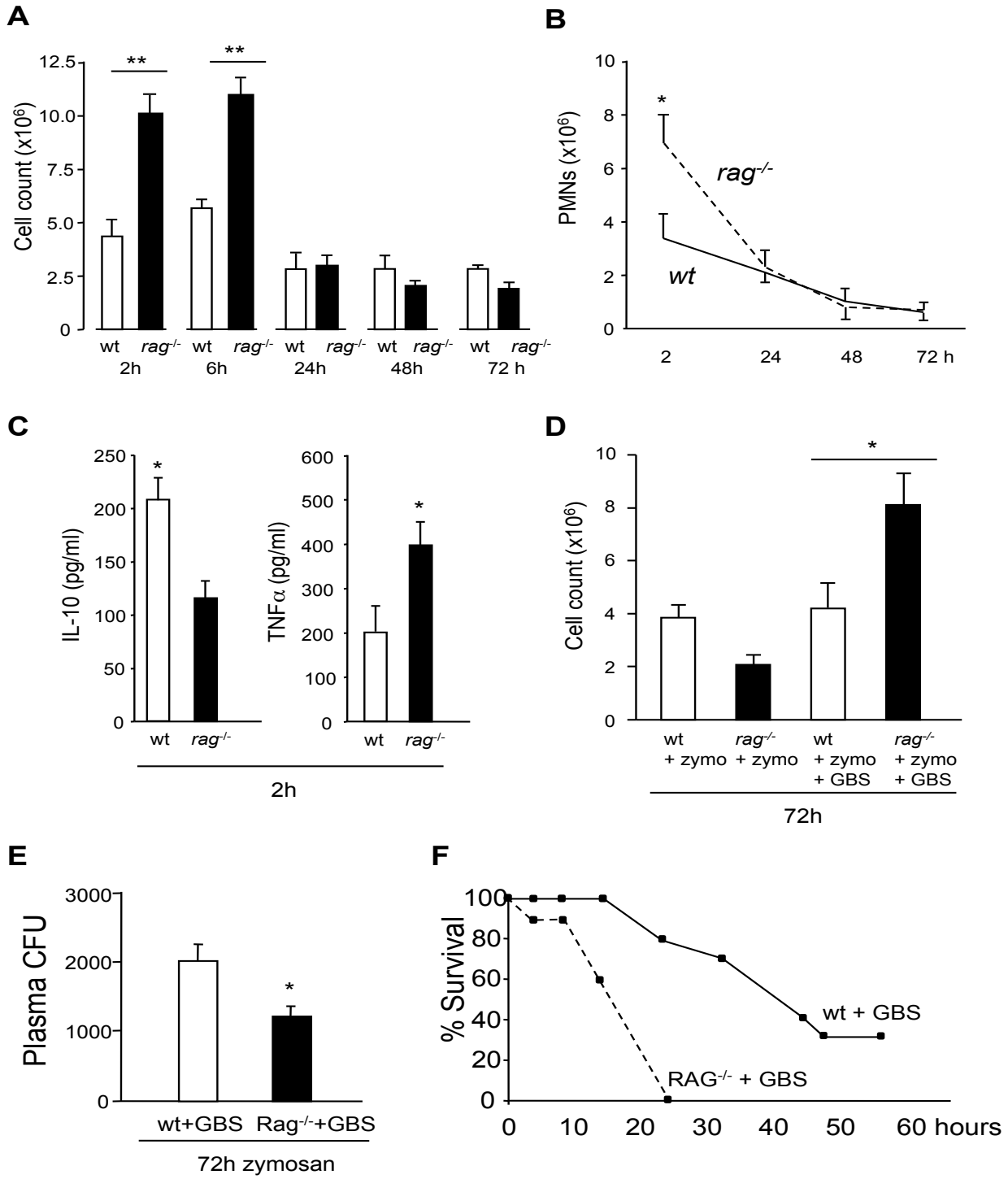


Figure 4.5 Zymosan peritonitis in lymphocyte-deplete RAG1$^{-/-}$ mice. Figures A-B Inflammation in RAG2$^{-/-}$ compared to wild type. C- Imbalance of IL-10 versus TNF. D- Cell numbers when RAG2$^{-/-}$ and wild types were injected with GBS during resolution (48 h after zymosan injection). E- Reduced bacterial colonisation in plasma but increased mortality in RAG1$^{-/-}$ mice (F). *n* = 6-8 animals per group with experiments repeated on two separate occasions to confirm original findings; *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA, followed by Bonferroni test, with data expressed as mean ± SEM.

4.3.5 Deficiency of repopulating lymphocytes in non-resolving chronic granulomatous disease

Finally, the relevance of these findings to human inflammatory diseases was determined by examining the role of lymphocytes in gp91^{phox} knockout mice, an experimental model of human chronic granulomatous disease caused by defects in the phagocyte respiratory burst oxidase, which generates microbicidal superoxide (Segal, Geisow et al. 1981; Dinauer 1993). Hence, chronic granulomatous disease patients lack antimicrobial capacity and the ability to combat bacterial and fungal infections. Moreover, this defect is associated with inflammatory granulomas in lung, liver and skin, which, in some instances may arise from sterile stimuli suggesting that their formation may be due to incomplete degradation of inflammatory debris and/or impaired resolution of inflammation (Gallin, Buescher et al. 1983; van de Loo, Bennink et al. 2003). gp91^{phox} knockouts were injected i.p. with sterile zymosan and found to have elevated leukocyte numbers compared to controls, with inflammation failing to resolve (Figure 4.6A). Fewer lymphocytes were found at 48 h–96 h in gp91^{phox} knockout mice, the time-frame of resolution and lymphocytes repopulation in wild types (Figure 4.6B). This was confirmed on FACS analysis (Figure 4.6C). Lymphocytes obtained from the resolution phase (72 h) of normal strain-matched wild type controls and therefore comprising B1 cells, NK and gamma/delta T cells as well as $CD4^+/CD25^+$ cells were transferred back to gp91^{phox} knockouts (72 h) and challenged with LPS. Inflammation was reduced in knockouts that received resolution-phase lymphocytes compared to gp91^{phox} mice alone, Figure 4.6D. These results in a chronic inflammatory model, suggest that during on going, non-resolving inflammation the absence of lymphocytes may account for susceptibility to super-infection and the associated hyper-inflammatory response.

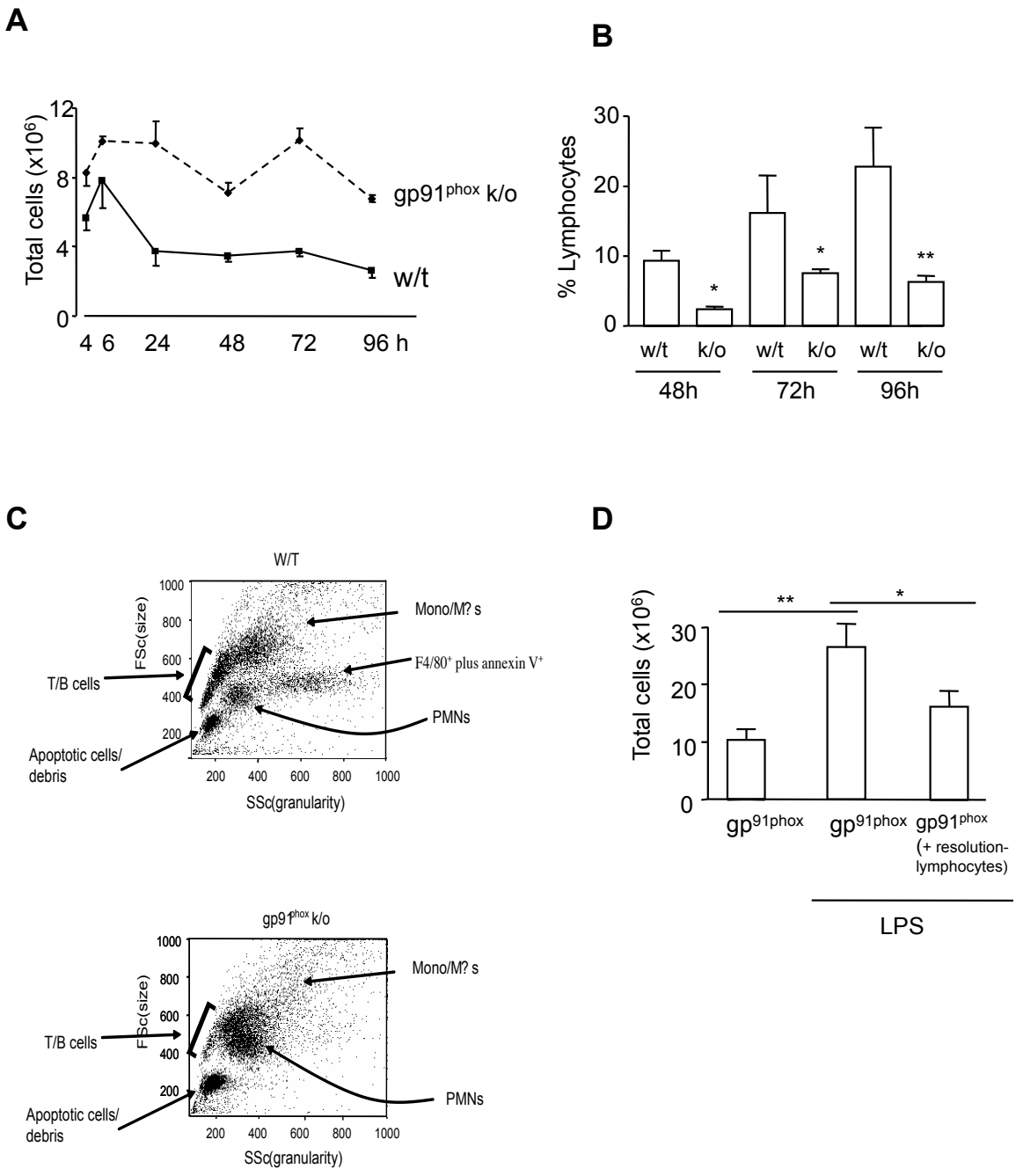


Figure 4.6 The absence of repopulating lymphocytes during non-resolving inflammation. Figure A- Zymosan was injected into the peritoneal cavity of gp91^{phox} knockout mice, which, when compared to controls showed a more aggressive inflammatory response that failed to resolve. B- The lymphocyte number in gp91^{phox} and wild type mice during the course of peritonitis. Figure C- FACS analysis of cell types present during resolution gp91^{phox} knockout and wild type mice. D- Effect of adoptive transfer of lymphocytes obtained from the resolution phase (72 h) into the peritoneal cavity of gp91^{phox} knockouts (72 h) and subsequently challenged, intraperitoneally, with LPS. *, $P \leq 0.05$; **, $P \leq 0.01$ as determined by ANOVA, followed by Bonferroni test, with data expressed as mean ± SEM.

4.3.6 Conclusions

1. In the naïve mouse peritoneal cavity, lymphocytes constitute half the cell population of which approximately 70-80% are B lymphocytes, of B1 subset.

2. In both human and murine peritonitis, peak inflammation is characterized by peak influx of PMNs and depressed lymphocyte numbers, which then re-populate the peritoneal cavity during resolution.

3. Exacerbation of murine peritonitis with higher doses of zymosan lead to persistently decreased number of lymphocytes. This is also mirrored in sterile peritonitis in a murine model of CGD, NADPH oxidase knockouts ($gp91^{phox}$ knockout mice).

4. Both B and T lymphocytes re-populate during the resolution phase.

5. During resolution

    a. There is a greater number of $CD4^+CD25^+$ and gamma-delta T cells as well as NK cells

    b. $CD5^+$ B-1 cells which comprise of over 80% of naïve B cells, re-populate the resolving peritoneal cavity with greater MAC-1 expression

6. The disappearance of B cells during peak inflammation is PGD_2 receptor DP-1 dependent

7. Re-populating lymphocytes protect the resolving peritoneal cavity against secondary infection based on the exaggerated inflammation and lethality when lymphocyte deplete RAG1-/- mice were challenged with GBS during resolution from zymosan peritonitis

4.4 Discussion

The evidence presented shows that lymphocytes play a pivotal role in controlling the onset of innate immune-mediated inflammation as demonstrated by the exaggerated response to zymosan in lymphocyte deficient $RAG1^{-/-}$ mice by regulating cytokine synthesis. It also has an important role in host susceptibility to secondary infection. Analysis of lymphocyte subsets in the naïve peritoneal cavity of mice revealed that B cells constitute about 70-80% of the total lymphocyte population of which the majority have a B1 phenotype with the remainder being B2 cells. CD3 positive cells as well as NK and gamma/delta cells make up the remaining 20% of lymphocytes. This profile differs to that found at resolution, which comprises more innate-type lymphocytes and B1 cells expressing MAC-1. It was no clear which lymphocyte or combination of lymphocytes bestowed protection at onset or at resolution. In the previous chapter peritoneal CD3 T cells and in particular B220 positive B cells were found to elaborate high levels of IL-10 in a DP1 dependent manner. This may explain the reduction in IL-10 in lymphocyte deficient $RAG1^{-/-}$ mice and subsequent increase in PMN influx in these animals. Thus, given the relative proportion of T cells versus B1 cells in the inflamed cavity and the ability of B cells to synthesize comparatively high levels of IL-10 we suspect that B lymphocytes may one of the predominant cell types modulating acute inflammatory responses to non-specific stimuli.

As PMNs begin to accumulate in response to zymosan, B cells disappeared in a PGD_2-dependent manner as there was equivalent numbers of B cells in the inflamed cavity of $hPGD_2S^{-/-}$ at 6h as there was in the naïve cavity of wild types. This accumulation of B cells in $hPGD_2S$ knockouts was reversed by BW245C, a DP1 receptor agonist with DP2 (CRTH2) playing no role in this setting. While the mechanism of PGD_2-dependent B cell clearance is unknown, a TLR-mediated transient down-regulation of integrins and CD9 on B1 cells was shown to be required for detachment of these cells from local peritoneal matrix and their subsequent efflux from the inflamed cavity (Ha, Tsuji et al. 2006). Furthermore B1 cell renewal is dependent on IL-5 and

recent work has identified a new lymphoid subset called fat associated lymphoid cluster (FALC) that stimulate B1 cell proliferation by producing IL-5. Whether BW245C alters CD9 expression needs further investigation but given that B cells disappear concomitantly with PGD_2 synthesis, it is possible that DP1 activation may play a role in regulating this pathway of B1 cell detachment and efflux. The fate of CD3 cells, on the other hand, is less clear. While their attachment to the peritoneal lining can certainly be excluded, there is the possibility that these cells may die locally by programmed cell death in response to 15d-PGJ_2, which is synthesized concomitant with their disappearance (Chapter 3, Figure 3.3B) and a potent inducer of lymphocyte apoptosis (Chen, Shen et al. 2005). However, future detailed work is required to definitively identify whether they die locally or clear *via* draining lymphatics. Thus PGD_2 exerts a dual role on resident B cells at least – regulating their inflammatory cytokine release as well as their efflux from the inflamed peritoneal cavity.

The mechanism by which lymphocytes exert their protective effects in these experiments is unclear. Certainly, there is a cytokine imbalance favouring pro-inflammatory TNFα but reduced IL-10 in RAGs. This may help explain the enhanced influx of PMNs compared to wild types in T/B cells deficient mice. Indeed, in chapter 3, I have shown that both T and B lymphocyte are capable of elaborating inflammatory cytokines, which, in the inflamed peritoneum at least, serve to limit PMN influx. However, in addition to cytokines/chemokines, cell adhesion molecules facilitate PMN adhesion and accumulation at sites of inflammation. The protective effect of resolution phase lymphocytes demonstrated in this study is in contrast to the finding of the presence of effector memory T cells in peritoneal dialysis patients with increased expression of pro-inflammatory chemokine receptor CCR5 (Roberts, Baird et al. 2009). Furthermore, there is evidence showing that lymphocytes trigger cell adhesion molecule expression. For instance, intracellular adhesion molecule 1 expression in *Plasmodium*-infected mice is reduced in the brain but not the lung of $RAG1^{-/-}$ mice while P-selectin expression is attenuated in both organs in

these animals (Bauer, Van Der Heyde et al. 2002). Equally, T cells were shown to enhance the expression of TNFα-triggered endothelial cell adhesion molecule expression, with these effects varying between vascular beds (Horie, Chervenak et al. 1997). However in this study the RAG2 mice are sterile and do not possess immunological memory as opposed to dialysis patients who develop recurrent infections which may explain the difference. Furthermore, it is important to highlight the different profile of lymphocytes present in the peritoneum (B1, B2 and small numbers of CD4/CD25 cells) that exert a predominantly protective effect in both the naïve and post-resolution state.

The trigger for lymphocyte repopulation is unclear but critical determinants of resolution such as PMN apoptosis or signals released by macrophages during phagocytosis of apoptotic leukocytes may play a central role. However, we have shown previously that inducible cyclooxygenase is expressed during and is essential for the resolution of acute inflammation (Gilroy, Colville-Nash et al. 1999; Gilroy, Colville-Nash et al. 2003; Gilroy, Newson et al. 2004), while others have reported that lipoxygenase-lipoxygenase interaction products of arachidonic as well as eicosapentaenoic and docosahexanoic acid dampen the severity of inflammatory onset and trigger resolution (Ariel, Fredman et al. 2006; Schwab and Serhan 2006; Schwab, Chiang et al. 2007). Taking a closer look at whether cyclooxygenase or lipoxygenase play a role in lymphocyte re-population, we found that not only is COX 2 expressed during the resolution phase of zymosan-induced peritonitis but that its inhibition with either NS-398 of the non-selective COX inhibitor indomethacin, impairs lymphocyte repopulation, in particular CD3 positive cells.

Comparing repopulating lymphocytes with those in the naïve cavity revealed more NK cells, gamma/delta T cells and $CD4^+/CD25^+$ cells in addition to B1 cells with higher Mac-1 labelling than that found at onset. The functional relevance of increased Mac-1 expression on resolving B1

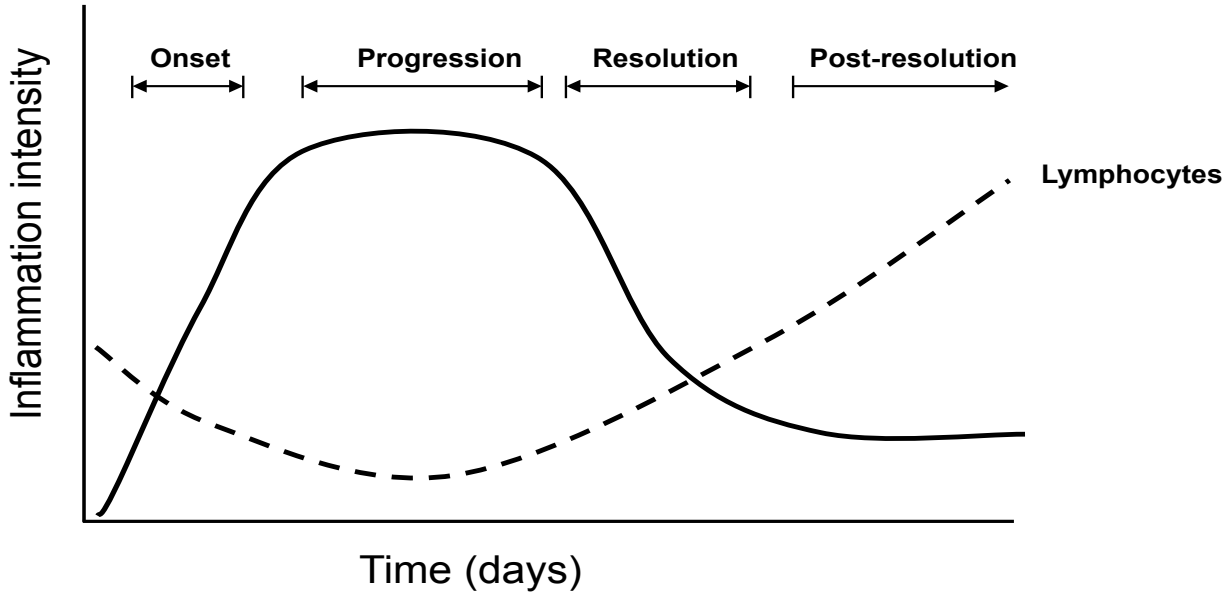
cells is not apparent at this stage but may reflect a state of differentiation/activation or a specific requirement for migration back to the resolved peritoneum. However, re-populating lymphocytes have no role in actively bringing about resolution but protect against super-infection. In these experiments of innate inflammation, GBS was given into the resolving peritoneal cavities of wild types as well as $RAG1^{-/-}$ mice. Interestingly, the degree of inflammation in wild types that received zymosan followed by GBS was not significantly greater than the level of inflammation in resolving wild types not given GBS. In contrast, the degree of inflammation in $RAG1^{-/-}$ mice that received zymosan followed by GBS was almost twice that of inoculated wild types. This suggests that resolution-phase lymphocytes confer protection against secondary infection. This was confirmed by injecting live bacteria to $RAG1^{-/-}$ mice undergoing resolution, which subsequently died faster than wild types treated in the same manner (Figure 4.5F). Interestingly, in lymphocyte deficient $RAG1^{-/-}$ when acute lung injury with LPS was studied, peak inflammation was no different compared to wild type but resolution was delayed, which was normalized with $CD4^{+}CD25^{+}Foxp3^{+}$ cells. Therefore, in order to work out the critical determinants that confer protection from secondary infection adoptive transfer experiments need to be carried out with subpopulation of lymphocytes i.e. B1, B2, and CD4+CD25+. It is also unknown why the proportion and profile of repopulating lymphocytes is different to that at onset. Perhaps as resolving tissues are physiologically altered as a consequence of the inflammatory event they underwent, host defence mechanisms need to be fundamentally different to guard against super-infection by recruiting more protective lymphocytes. On this theme, it is not really understood at which point of the inflammatory cascade acutely inflamed tissues revert back to their original state. Certainly, a population of macrophages (about $1x10^{6}$) were found to linger for at least three weeks after zymosan peritonitis apparently resolved peritoneal cavity, supporting the idea that although the original response was acute and transitory in terms of PMN influx and efflux, its effects may be longer lasting than originally believed. This may explain a different profile and greater proportion of lymphocytes that assist in modulating future

inflammatory events. This may explain why inflammation in the resolving phase conferred greater protection against GBS lethality than un-inflamed or naïve mice (Figure 4.5D and F).

Taking these findings to a more clinically relevant setting, it became clear that unlike wild type mice, there was a deficit of repopulating lymphocytes in non-resolving gp91^{phox} knockouts mice bearing zymosan-induced peritonitis. Replenishing gp91^{phox} knockouts with resolution-phase lymphocytes taken from strain-matched controls and then challenging animals with LPS conferred protection compared to sham operated gp91^{phox} knockouts mice. Data from this study confirms that not only does lymphocyte repopulation fail to occur in non-resolving inflammation but that resolving-phase lymphocytes protect against exaggerated inflammatory responses to super-infection (Figures 4.5D and 4.6D). On this note, defects in innate lymphocyte functioning have been suggested to lead to secondary infections associated with burn injury (Schneider, Glenn et al. 2007) while increased lymphocyte apoptosis contributes to the pathogenesis of sepsis (Hotchkiss, Tinsley et al. 1999; Hotchkiss, Chang et al. 2000) underlining the crucial role lymphocytes play in host defence against non-specific injury. Moreover, as NK cells were originally described for their ability to lyse tumour cells (Moretta, Bottino et al. 2002) and gamma/delta T cells have well-known tumour surveillance properties (Carding and Egan 2002; Hayday and Tigelaar 2003), their absence from sites of non-resolving inflammation may be one of the predisposing factors to the development of inflammation-related cancer (Lin and Karin 2007). Thus, one of the hazards of on going acute inflammation and consequently failed lymphocyte repopulation may be increased susceptibility super-infection and even cancer as a result of lymphocyte apoptosis, lymphocyte immuno-paralysis or as presented here, a failure of protective lymphocytes to repopulate post resolution.

In summary, this study demonstrates a biphasic role for lymphocytes during innate immune-mediated inflammation, summarized in Figure 4.7. The first phase controls PMN trafficking with

lymphocytes then vacating the peritoneal cavity in response to PGD_2 activating its DP1 receptor. The second phase is characterized by lymphocyte repopulation occurring after inflammation begins to resolve in a different proportion and profile to that of the naïve state. Importantly, repopulating lymphocytes have no role in bringing about resolution but protect against secondary infection.



Onset

CD3 (CD4/CD8)

$CD4^+/CD25^+$

NK ↑

Gamma/Delta T cells ↑

B1 ($B220^{low}/Mac\text{-}1^{low}/CD5^+$)

B2 ($B220^{high}/Mac\text{-}1^-$)

(Control PMN trafficking)

Post-resolution

CD3 (CD4/CD8) ↑

$CD4^+/CD25^+$ ↑

NK ↑↑↑

Gamma/Delta T cells ↑↑↑

B1 ($B220^{low}/Mac\text{-}1^{high}/CD5^+$)

B2 ($B220^{high}/Mac\text{-}1^-$)

(Protect against 2nd infection)

Figure 4.7 A summary of the scheme of events that occurs in acute inflammation with reference to lymphocyte trafficking. As inflammation ensues resident lymphocytes begin to disappear, with B1 cells clearing *via* draining lymphatics with the fate of CD3 cells remaining unclear at this stage. Once inflammation begins to resolve lymphocytes re-populate the site of injury in a profile different to that in the naïve state.

Chapter 5 The profile of adenosine and its metabolites in peritoneal inflammation of gp91phox$^{-/-}$ mice, a model of non resolving inflammation

5.1 Introduction

Failed resolution is hypothesised to lead to tissue injury and chronic inflammation (Lawrence and Gilroy 2007). This is potentially derived from dysregulated resolution pathways of which CGD is a good example. This is an inherited immunodeficiency syndrome caused by a defect in the oxygen metabolic-burst machinery resulting in the inability to neutralise infection leading to persistent and recurrent inflammatory responses and granulomatous tissue formation (Segal 1996). Activity of NADPH oxidase system ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$) is either absent or dysregulated in these patients with the most common being X-linked CGD (~65%) with defects in the gene encoding $gp91^{phox}$. Fortunately, for the purpose of understanding the aetiology of CGD, $gp91^{phox}$ deficient mice display all the hallmarks of the human condition in response to infection (Pollock, Williams et al. 1995). Interestingly, there are reports showing that inflammation in CGD mice is also prolonged and dysregulated in response to sterile stimuli, suggesting potential irregularities in endogenous anti-inflammatory and/or pro-resolution pathways (Morgenstern, Gifford et al. 1997; van de Loo, Bennink et al. 2003). I carried out a series of studies in $gp91^{phox-/-}$ mice and found that of the factors known to control resolution, few appeared to be consistently dysregulated with the exception of adenosine and cAMP, levels of which are significantly lower in experimental CGD compared to wild types.

Under physiological conditions, adenosine is continuously formed both intra and extracellularly. The intracellular production is mediated either by 5'-nucleotidase, which dephosphorylates AMP or by hydrolysis of *S*-adenosyl-homocysteine (Fredholm, AP et al. 2001). Adenosine generated within cells is transported into the extracellular compartment *via* bi-directional transporters through facilitated diffusion that efficiently equilibrates intra and extracellular levels of adenosine. Following trauma, there is a decrease of intracellular ATP, accompanied by an accumulation of 5'-AMP and subsequently adenosine by the above pathways, which may be sequentially metabolized to inosine, hypoxanthine and xanthine. Expressed on cells of the

hematopoietic system, adenosine receptors (A_1, A_{2A}, A_{2B}, A_3) belong to the family of G-protein-coupled heptahelical transmembrane receptors, which either stimulate (Gs) or inhibit (Gi) adenylyl cyclase, the enzyme that catalyzes the formation of cAMP (Fredholm, AP et al. 2001). Adenosine A_1 and A_3 receptors are high and low affinity receptors for adenosine, respectively, with both being inhibitors of adenylyl cyclase. High-affinity A_{2A} and low-affinity A_{2B} receptors, on the other hand, activate adenylyl cyclase, thereby increasing intracellular levels of cAMP, resulting in potent immune-suppression and regulation of inflammatory leukocyte trafficking. Besides controlling adenylyl cyclase, adenosine receptors are also coupled by distinct G-proteins to several other effector systems, including calcium and potassium channels, phospholipase C, D, A2, cGMP, phosphodiesterases, and mitogen-activated protein kinases that modulate different cell functions. Thus, adenosine, released after tissue injury or low oxygen tension associated with inflammation, has been regarded by some to act as a first line sensor of immune damage where it prevents further damage by inhibiting activated immune cells with its immune-suppression mediated by A_{2A} receptor elevation of cAMP (Hasko and Cronstein 2004; Sitkovsky and Lukashev 2005).

In this chapter, the biphasic synthesis of both adenosine and cAMP, first at the traditional early onset phase of acute inflammation and again during resolution was demonstrated, with synthesis of these immunosuppressive agents being significantly lower in CGD ($gp91^{phox-/-)}$ mice associated with a severe and prolonged innate immune response to a sterile stimuli. We also show that hyper-inflammation in $gp91^{phox-/-}$ mice can be rescued by A_{2A} receptor activation as defined by reduction in inflammatory leukocytes. Importantly, A_{2A} receptor activation in $gp91^{phox-/-}$ mice did not bring about resolution as this drug strategy was not associated with innate-type lymphocyte repopulation that is typical of events that occur during normal resolution in wild type leading to tissue homeostasis.

5.2 Material and Methods

The detailed material and methods are described in chapter 2. Peritonitis was induced in gp91$^{phox-/-}$ and wild type mice with intra-peritoneal zymosan 1mg following pre-treatment with the A_{2A} receptor agonist CGS 21680 (2 mg/kg) with/without ZM241385 (2 mg/kg, A_{2A} receptor antagonist) 30 min previously. The peritoneal cavity was lavaged at 4 h and cell number counted using a haemocytometer. For experiments in the resolution phase, inflammation was firstly induced with zymosan followed by CGS 21680 or vehicle (DMSO) at 24 and 36 h. The peritoneal cavity was lavaged at 48 h following zymosan. Caffeine as well as its stable analogue, 8-(3-chlorostyryl)-caffeine (CSC) was dosed at 3 mg/kg. For eicasonoid analysis, the samples stored at -20^{0}C were thawed at room temperature and acidified to pH 3. Samples were extracted using C18 columns (Waters). For PGD_2, samples were then treated with methoxylamine hydrochloride (MOX HCL) and the resulting stable PGD_2-MOX measured by EIA. PGE_2 was measured by EIA (Cayman Chemicals) while lipoxin A4 was quantified by ELISA (Neogen Europe, Ayr, UK). Proteins in exudates were removed by ultrafiltration (30,000 Da cut-off). Purine concentrations in samples were measured by HPLC carried out in the laboratory of Professor Edwin Jackson at University of Pittsburgh Medical Centre, Pittsburgh, USA. Cytokines were measured by ELISA as per manufacturer instructions. Leukocytes were incubated with antibodies for 30 min to either CD3/CD19 (Abd Serotec, Oxford UK), B cells (Ly220, Abd Serotec, Oxford UK), GR1 (BD Pharmingen, UK) or F4/80 (Caltag laboratories, CA, USA) using respective isotype antibodies as controls (Abd Serotec, Oxford UK) and compensated as appropriate for dual labelling. For apoptosis, cells were incubated with annexin V/PI (Becton Dickinson) and analysed on Becton Dickinson FACScalibur with data analysed by Cellquest.

5.3 Results

5.3.1 Exaggerated inflammatory response in gp91$^{phox-/-}$ mice

Zymosan injection into the mouse peritoneal cavity of gp91$^{phox-/-}$ mice and wild type controls resulted in an exaggerated inflammation (Chapter 4, Figure 4.6A) with significantly lower lymphocyte numbers (Chapter 4, Figure 4.6B). In the acute phase there was a significant excess of PMNs (Ly6G positive cells) in knockout animals with numbers declining up to 96 h (Figure 5.1A). F4/80 positive macrophage numbers were similarly increased at onset in knockouts with levels remaining elevated throughout the entire response (Figure 5.1B), underlining the non-resolving nature of inflammation that is characteristic of CGD. As there was little difference in leukocyte apoptotic rates as determined by annexin V/PI labelling between both animals, Figure 5.1C, failure of resolution in gp$^{91phox-/-}$ mice most likely resulted from continual influx and/or failed clearance of inflammatory leukocytes.

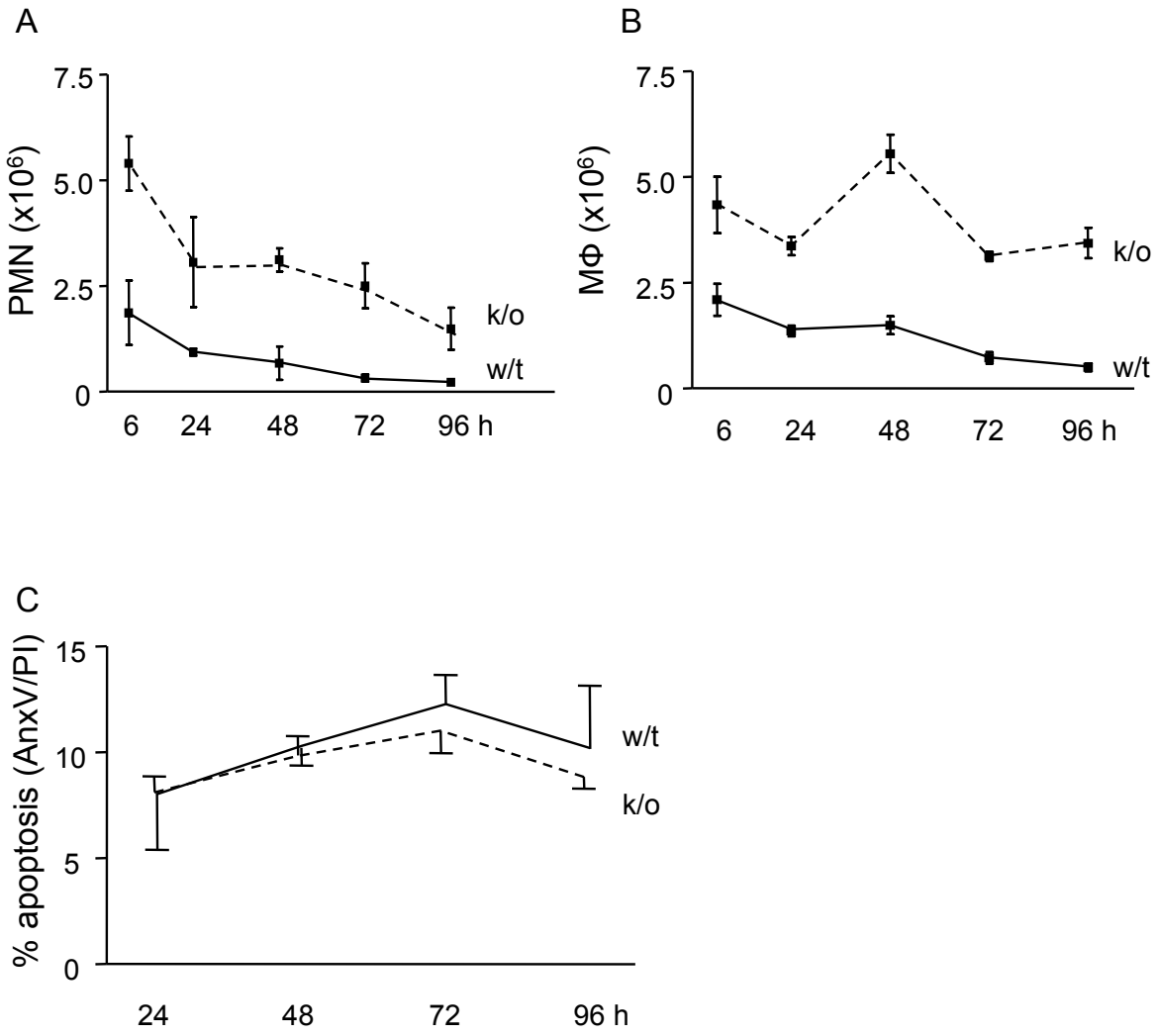


Figure 5.1 Zymosan induced peritonitis in gp91$^{phox-/-}$ and wild type C57black VI mice. Figures A -B- Inflammation is more severe and fails to resolve in gp91$^{phox-/-}$ mice with more exaggerated influx of Ly6G-positive PMNs in gp$^{91phox-/-}$ mice along with F4/80-positive monocyte-derived.

5.3.2 Reduced synthesis of purines and cAMP in gp$^{91phox-/-}$ mice

The exaggerated inflammatory response in CGD is explained by defects in phagocytic oxidase resulting in impaired bacterial killing and consequently delayed removal of the injurious agent. However for resolution to occur there are endogenous braking systems that counter-regulate acute inflammation. Therefore, I screened for alterations in levels of anti-inflammatory and pro-resolution mediators in gp91$^{phox-/-}$ and wild type mice bearing a zymosan-triggered peritonitis was studied. With the exception of PGE_2 (Figure 5.2C), there was little evidence for defects in

arachidonic acid metabolism being involved in the inflammatory profile of CGD, with no significant difference in PGD_2 (Figure 5.2A) and Lipoxin A4 (Figure 5.2B) in $gp91^{phox-/-}$ and wild type mice with zymosan induced peritonitis. However, there was a significant reduction in levels of cAMP in $gp91^{phox-/-}$ mice compared to wild type animals (Figure 5.2D). Specifically, intracellular cAMP was elevated during the early onset phase of zymosan-induced peritonitis in wild type controls (4-6 h), waning as inflammation progressed and became elevated again post-resolution (Figure 5.2D). In $gp91^{phox-/-}$ mice bearing a zymosan-triggered peritonitis, cAMP was significantly lower than wild types at onset and failed to show the post-resolution elevation seen in wild types (Figure 5.2D). The biphasic profile of cAMP in wild type mice and significantly lower levels in $gp91^{phox-/-}$ mice was not due to PGD_2, $LX\text{-}A_4$ or PGE_2 the latter which in fact was elevated in the knockouts. However, there was a biphasic profile of adenosine synthesis mirroring that of cAMP – being raised at onset and then again at resolution (Figure 5.2E). Adenosine showed lower levels in $gp91^{phox-/-}$ mice than wild types and is a well described immuno-modulator of acute inflammation serving to dampen PMN function and prevent chemical-induced collateral liver injury. Therefore these studies show a biphasic synthesis of adenosine and cAMP in resolving models of acute inflammation, with significantly reduced levels when zymosan peritonitis was induced in $gp91^{phox-/-}$,a model of CGD.

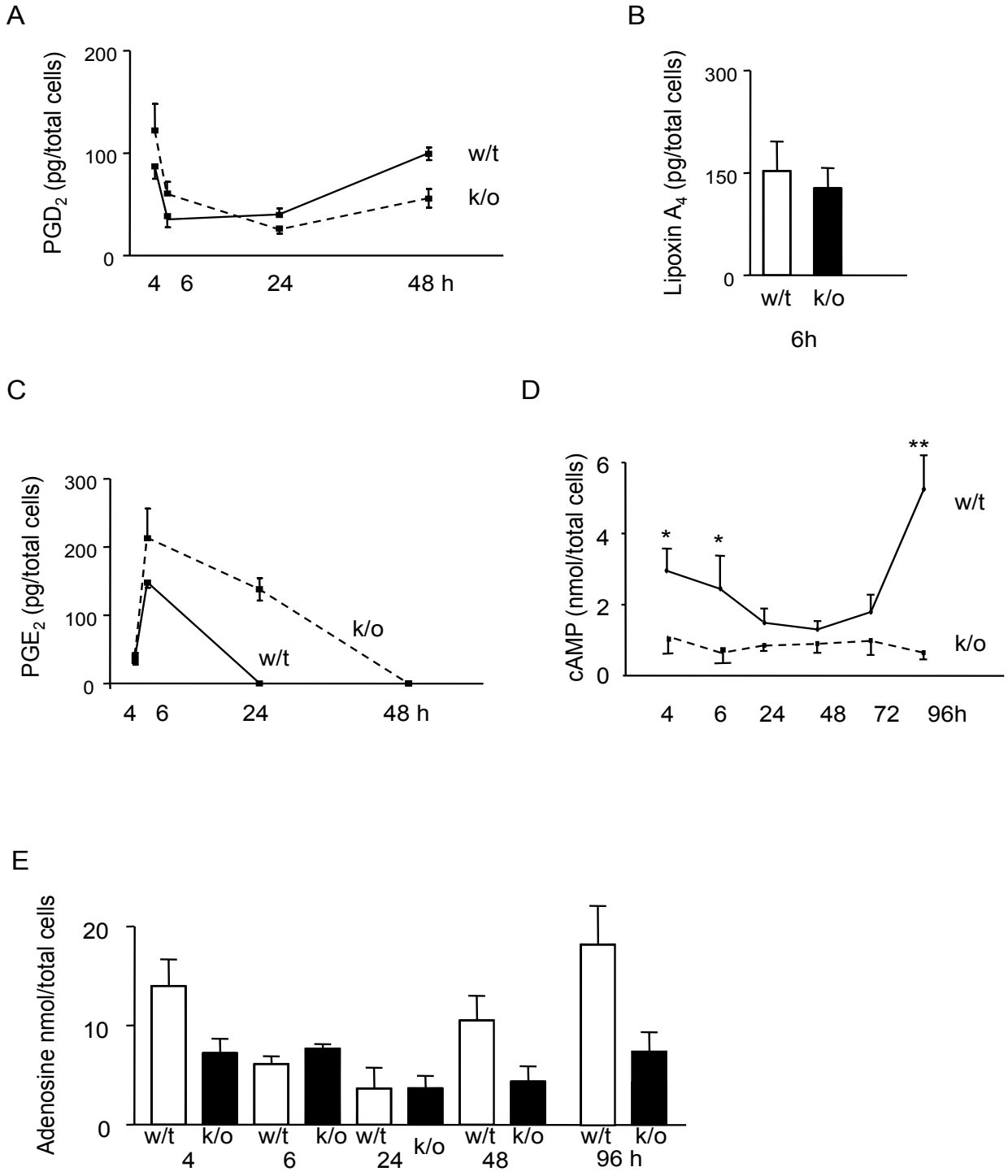


Figure 5.2 Arachidonic acid and adenosine metabolism in gp91$^{phox-/-}$ mice compared to wild types. Figures A-C PGD_2, lipoxin and PGE_2 were determined and found not to show a consistent reduction in gp91$^{phox-/-}$ mice compared to wild types. D- cAMP was elevated at the early onset phase of a murine peritonitis and then again at resolution in wild type animals, but was significantly lower in gp$^{91phox-/-}$ mice at both phases. E- Adenosine measured following zymosan peritonitis with levels being reduced in gp91phox$^{-/-}$ mice (black columns) compared to wild types (empty columns). *n* = 6–10 animals per group. *P*≤0.01; **, *P*≤0.001, as determined by ANOVA, followed by Bonferroni test, with data expressed as mean ± SEM.

5.3.3 Adenosine and cAMP profiles in human resolving peritonitis

In the previous chapter, when the inflammatory cell profile of CKD stage 5 patients on peritoneal dialysis who developed peritonitis were studied, the cell numbers were greatest at the start which was PMN rich (day 1) and resolution by day 5 with lymphocyte re-population (Chapter 4, Figure 4.1A-C). cAMP (Figure 5.3A) as well as adenosine, (Figure 5.3B) were measured in these samples and the levels were elevated as inflammation resolved. While these results corroborate that found in mice, we have no data on the very early onset (~ 6 h) phase in humans to assess whether the adenosine and cAMP in human inflammation mirror the biphasic profile found in murine peritonitis. However, as in rodents, levels of cAMP and adenosine were elevated as inflammation resolved.

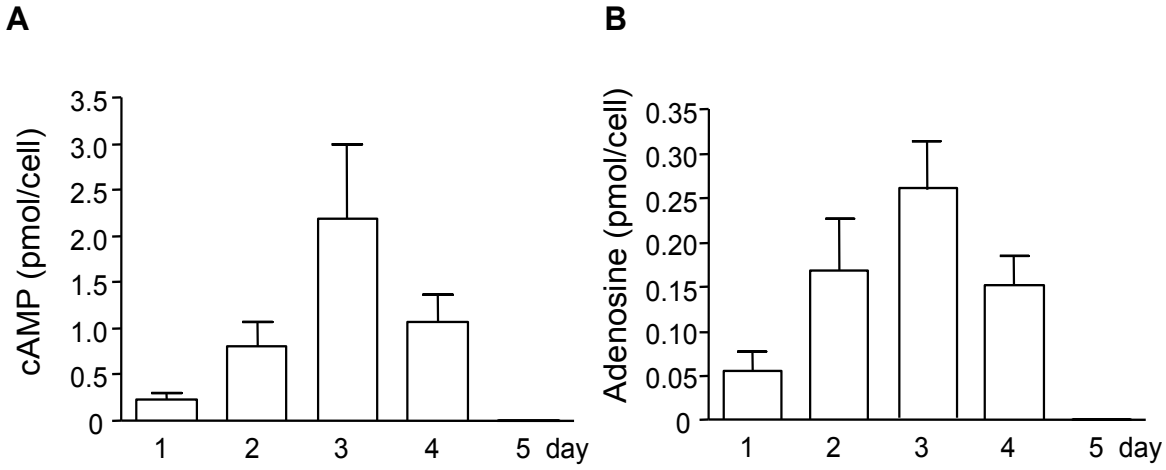


Figure 5.3 Expression of adenosine and cAMP concomitant with resolution of human peritonitis. Figures A-B- Analysis of cell-free exudates revealed a peak in cAMP and adenosine as inflammation resolved in peritonitis samples obtained from patients undergoing chronic ambulatory peritoneal dialysis and who experienced transient infection that resolved within a few days. n= 6-8 patients with data expressed as mean ± SEM.

5.3.4 Adenosine, *via* A_{2A} is anti-inflammatory and not resolution-toxic

Adenosine exerts its action through four receptors, A_1, A_2 (A_{2A} and A_{2B}) and A3. The A_{2A} receptor has received the most attention, as its activation leads to the up-regulation of cAMP and thereby mediating its anti-inflammatory effect. In rats bearing a carrageenin-induced pleurisy, when rolipram, a PDE4 inhibitor (elevator of cAMP) as well as CGS 21680, a specific A_{2A} receptor agonist were administered 30 min prior to carrageenin injection, it dampened leukocyte trafficking to the inflamed cavity at onset i.e. 4 h (Figure 5.4A). This was associated with an expected rise in cAMP, Figure 5.4B. Therefore, I proceeded to investigate this in the mouse zymosan peritonitis model. At 4 h following i.p. zymosan, CGS 21680 dampened inflammation in an A_{2A} receptor dependent manner as co-administration of the receptor antagonist ZM 241385 reversed the anti-inflammatory effect (Figure 5.4C). This was associated with a significant increase in anti-inflammatory IL-10 (Figure 5.4D). Other drugs, including theophylline as well as beverages are known to alter cAMP signalling and therefore may unwittingly affect inflammation depending on its effect of levels. A single cup of coffee, for instance, contains about 100 mg of caffeine implying that an average person drinking one cup of coffee per day will ingest caffeine at 1.5mg/kg. To investigate whether caffeine, a methylxanthine with antagonistic effects on the A_{2A} receptor (Fredholm, Battig et al. 1999) affects acute inflammation, caffeine as well as its stable analogue, 8-(3-chlorostyryl)-caffeine (CSC) at 3 mg/kg were administered 30 min before i.p. zymosan injection and found that both worsened inflammation possibly by decreasing protective IL-10 levels (Figures 5.4E and F). Using a continual pharmacological dosing regimen, mice were administered not only 30 min before zymosan injection but were also given caffeine and CSC again at 12 h and 18 h after zymosan and their effects assessed at 24 h. CGS 21680 maintained a dampening of acute inflammation without interfering with resolution (Figure 5.4G). On the other hand, caffeine at doses representative of reasonable caffeine daily intake as well as its analogue, CSC, maintained their

pro-inflammatory effects (Figures 5.4G). Taking the doses of caffeine up to 10 and 30 mg/kg resulted in a loss of pro-inflammatory effects.

Therefore from these experiments, A_{2A} agonists can be seen to exert anti-inflammatory effects not only during the early onset phase of acute inflammation, the phase traditionally tested experimentally for novel anti-inflammatories, but, importantly do not appear to interfere with pro-resolution pathways.

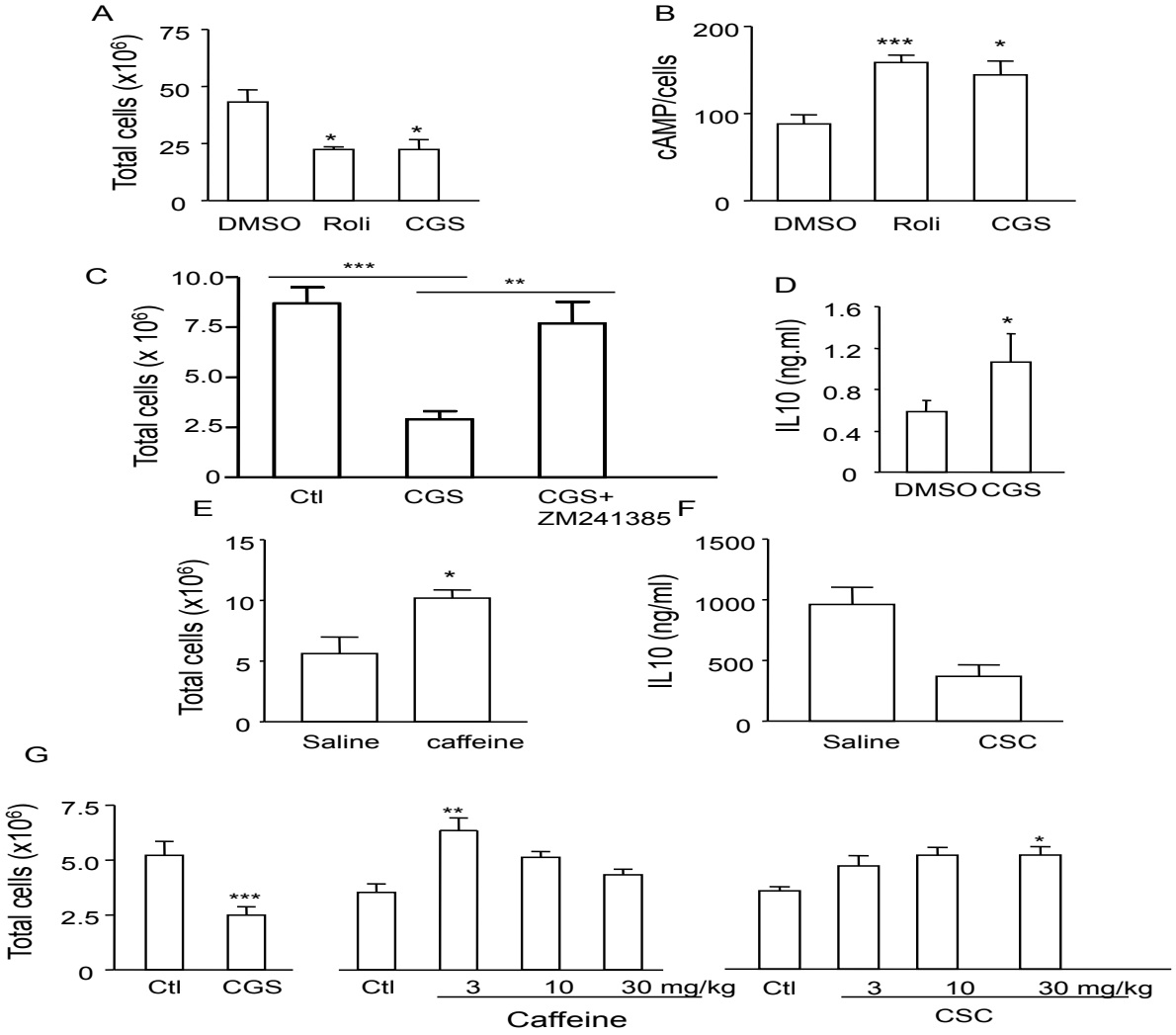


Figure 5.4 Investigation of Adenosine, *via* A_{2A} being anti-inflammatory and not resolution-toxic. Figures A-B- Effect of selective A_{2A} agonist CGS 21680 as well as the PDE4 inhibitor rolipram, which increases cAMP were dosed at 10 and 30 mg/kg respectively, to rats bearing a carrageenin-induced pleurisy. Figure C- A_{2A} receptor activation in a zymosan-induced peritonitis and the effects of CGS 21680 with or without ZM241385 (A_{2A} receptor antagonist) determined 4 h later revealing, again, that (C) CGS 21680 is anti-inflammatory in this model concomitant with an elevation in anti-inflammatory IL-10 (D). Figures 4E-G the A_{2A} receptor antagonist (E) caffeine and its (F) stable analogue, CSC worsened inflammation and depressed IL-10, respectively, when administered in a similar manner to receptor agonists. A_{2A} receptor activation maintained its anti-inflammatory effects and did not interfere with resolution pathways as injection of (G) CGS 21680, given 30mins before zymosan and again 12 h and 18 h later continued to dampen inflammation while caffeine and its analogue worsened the response as determined at 24 h. *n* = 8 animals per group; *, *P*≤ 0.05; **, *P*≤0.01, *** P<0.001 as determined by ANOVA, followed by Bonferroni test.

5.3.5 Anti inflammatory but not pro-resolution effect of adenosine *via* A_{2A} in gp91$^{phox-/-}$ mice

Being anti-inflammatory is not the same as possessing pro-resolution properties (Gilroy, Lawrence et al. 2004). The objective of this final set of experiments was to determine whether drugs that signal through A_{2A} and raise cAMP are able to rescue the hyper-inflammatory phenotype typical of gp91$^{phox-/-}$ mice and importantly, whether they bring about resolution of peritoneal inflammation. Thus, CGS 21680 was dosed orally 30 min before zymosan administration to gp91$^{phox-/-}$ and wild types with inflammation assessed 4 h later. Data revealed that leukocyte influx was greater in knockouts than wild types and that A_{2A} receptor activation in knockouts reversed inflammation back to levels seen in drug-treated wild types (Figure 5.5A), with lowering of PMN numbers, Figure 5.5B. The A2A receptor agonist, CGS 21680 was then administered to gp91$^{phox-/-}$ mice and controls during the equivalent of resolution in wild types. In wild types, CGS 21680, given at 24 h and again at 36 h after established inflammation had no effect on leukocyte numbers at 48 h i.e. CGS 21680 was neither anti-inflammatory nor resolution-toxic in normal animals. An identical dosing regime in knockouts revealed that CGS 21680 was not only anti-inflammatory in these animals but that it lowered inflammation below that of controls (Figure 5.5C) concomitant with an elevation of cAMP (Figure 5.5D). However this anti-inflammatory effect was not associated with lymphocyte re-population which is typical of innate resolution as described in the previous chapter.

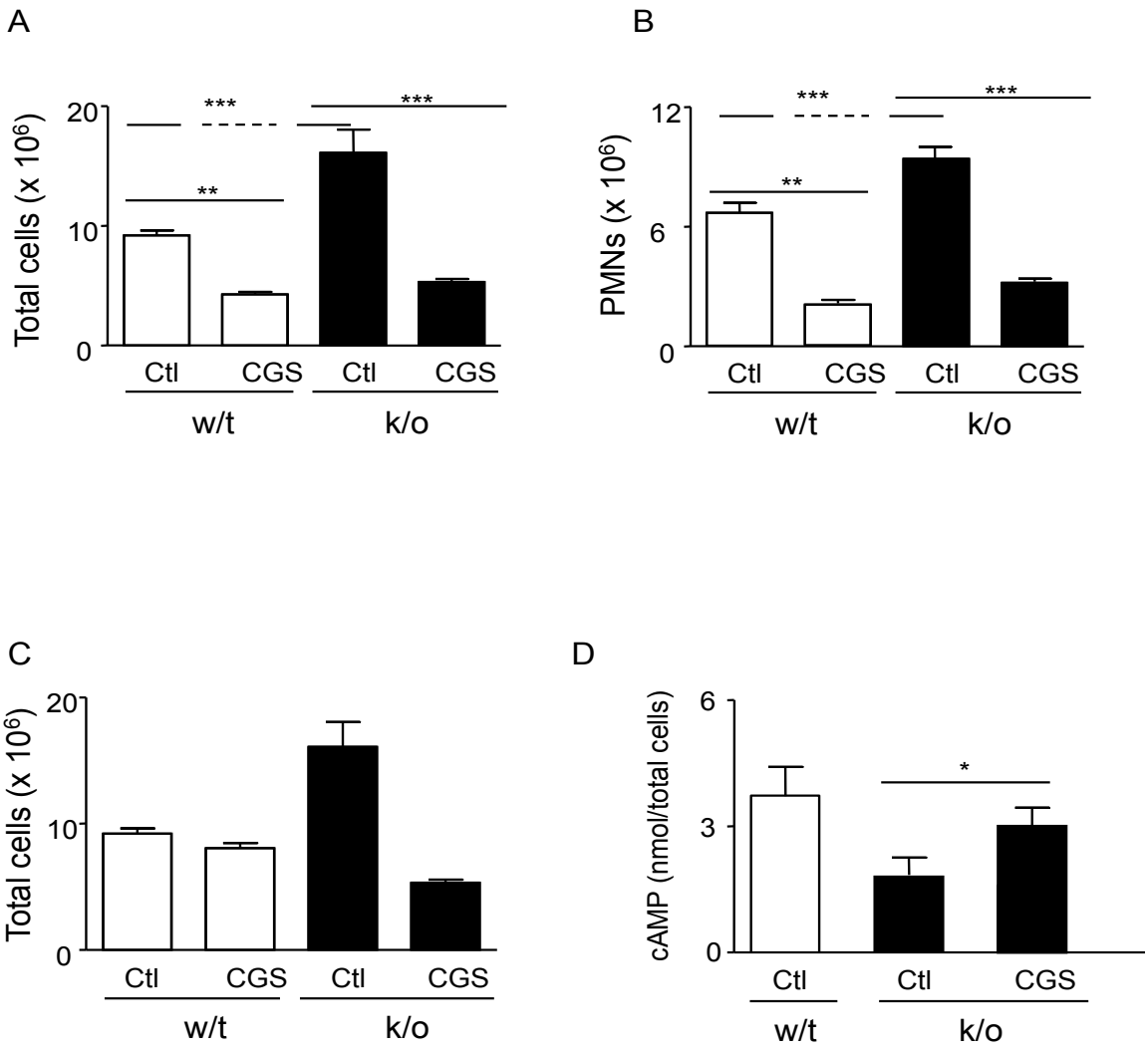


Figure 5.5 Demonstration of Adenosine, *via* A_{2A} being anti-inflammatory but not pro-resolution in gp91$^{phox-/-}$ mice. Figure A-B- Wild type and gp91$^{phox-/-}$ mice were injected with zymosan i.p. when pre-treated with A_{2A} agonist CGS 21680. CGS does rescue the hyper-inflammatory profile of knockout mice by the reduction of PMN numbers. Figure C-D Both administered to both animal types therapeutically i.e. 24 h and 36 h after zymosan and its effects on resolution and cAMP determined at 48 h. However there was an absence of lymphocyte re-population. *n* = 6 animals per group; *, *P*≤ 0.05; **, *P*≤0.01, as determined by ANOVA, followed by Bonferroni test, with data expressed as mean ± SEM.

5.3.6 Conclusions

1. In human and murine peritonitis both adenosine and cAMP demonstrate a biphasic response with levels raised at onset and during resolution of inflammation.

2. Peritoneal inflammation in NADPH gp91$^{phox-/-}$ mice was exaggerated compared to wild type with peak increase in PMN numbers and failure of resolution with persistent macrophage numbers

3. PGD_2 a product of the cyclooxygenase pathway of arachidonic acid metabolism is not involved in the increased inflammation or impaired resolution in this model of CGD

4. Both adenosine and cAMP were decreased in NADPH gp91$^{phox-/-}$ mice in zymosan peritonitis.

5. The acute hyper-inflammatory phenotype of NADPH gp91$^{phox-/-}$ mice was reversed by the A2A receptor agonist CGS 21680

5.4 Discussion

Here we report an immediate increase in adenosine and cAMP at the early onset phase of acute inflammation that wanes as the response progresses only to increase again as inflammation resolves with levels being significantly lower in experimental CGD ($gp91^{phox-/-}$ mice). Whether this is a direct result of an interaction between NADPH oxidase systems and adenosine synthesis is unlikely as levels of cAMP in isolated cells from CDG patients were similar, on a cell-for-cell basis, to normal healthy volunteers. The persistence of inflammation in CGD is either due to an inherent defect in counter-regulatory/pro-resolution pathways or more likely the failure to clear inflammatory stimuli resulting in a persistent, almost frustrated innate immune response that consequently nullifies endogenous protective pathways. As emphasised previously one of the most critical determinants for resolution of inflammation is clearance of the inflammatory stimulus (Gilroy, Lawrence et al. 2004; Serhan and Savill 2005). CGD is an example of where defects in clearance may be one of the primary causes of exacerbated and prolonged responses. Certainly, PMNs from CGD patients have impaired phagocytosis of immune-complexes while CGD macrophages are equally defective in their clearance of apoptotic PMNs (Brown, Goldblatt et al. 2003). Equally, the formation of granulomatous synovitis in response to intra-articular zymosan injection in NADPH oxidase-deficient mice was suggested to result from incomplete zymosan clearance from the joint due to impaired phagocytosis (van de Loo, Bennink et al. 2003). This, therefore, suggests that dysregulation in cAMP and adenosine is secondary to that of an overwhelming inflammatory event, whose pro-inflammatory signals deactivates or overrides endogenous anti-inflammatory and/or pro-resolution pathways.

From the above argument it should not be assumed that all endogenous protective pathways are depressed during CGD. Among some of the signals that counter-balance inflammatory onset and/or trigger resolution, neither PGD_2 nor native lipoxin A_4 levels were statistically different in $gp91^{phox-/-}$ mice compared to wild types at onset with the exception of PGD_2, which showed a

trend towards a reduction in knockouts as inflammation resolved. That notwithstanding, data presented here shows a more consistent dysregulation in the synthesis of adenosine/cAMP in $gp91^{phox-/-}$ mice in response to sterile zymosan. I went on to investigate and show that A_{2A} receptor activation rescues the hyper-inflammatory response in $gp91^{phox-/-}$ mice without subverting resolution in wild type animals. This latter point is important as existing anti-inflammatories, NSAIDS for instance, while being protective by virtue of their ability to dampen the early onset phase of acute inflammation, delay resolution and prolong inflammation (Gilroy, Colville-Nash et al. 1999). A_{2A} receptor activation, on the other hand, is anti-inflammatory without being resolution-toxic thereby displaying broader pharmacological flexibility and potentially fewer side effects in terms of prolonging inflammation. However, these data are counter-intuitive based on current understanding of cAMP in inflammatory leukocyte longevity and clearance as derived from *in vitro* studies. For instance, elevating cAMP in PMNs delays their apoptosis (Martin, Dransfield et al. 2001) while raising cAMP in monocyte-derived macrophages impairs their phagocytic capacity (Rossi, McCutcheon et al. 1998) suggesting that activating A_{2A} during inflammation and consequently elevating cAMP would lengthen the life span of PMN, impair their clearance and prolong inflammation. Despite these data from isolated cell systems, *in vivo*-derived data (Figures 5.4 and 5.5) clearly show that A_{2A} receptor activation is anti-inflammatory without being resolution-toxic, and that activation of this receptor at any phase of CGD dampens inflammation. This implies that CGD is in a constant state of perpetual acute inflammation and that A_{2A} receptors inhibit PMN influx. The current treatment regime for CGD patients is antibacterial and antifungal prophylaxis (Goldblatt 2002), but for exacerbations of inflammatory events, perhaps concomitant A_{2A} receptor activation would dampen associated inflammatory responses without subverting pro-resolution pathways. At the very least, such patients should avoid A_{2A} antagonist such as caffeine (and perhaps other dietary methylxanthines such as theobromine) as it may nullify whatever protection residual adenosine may confer in CGD during inflammatory events.

As mentioned previously, being anti-inflammatory in pharmacological terms is distinct from being pro-resolution (Gilroy, Lawrence et al. 2004). This was demonstrated in the previous chapter because in the resolving peritoneum not only there is a disappearance of PMN *via* apoptosis and macrophages *via* lymphatic drainage, but also the influx of innate-type lymphocytes. These repopulating lymphocytes do not switch off inflammation but modulate post-inflammatory responses to bacteria in the context of secondary infection. The lymphocytes disappear in response to inflammatory stimuli not before secreting cytokines that modulate the severity of the inflammatory response such that in $RAG1^{-/-}$ mice, for instance, inflammation is more exaggerated in terms of PMN trafficking. Therefore, as inflammation resolves, we suspect that repopulating lymphocytes simply reflect the inflamed tissue reverting to its prior physiological state under the control of as yet un-identified endogenous factors. Interestingly, we found no repopulating lymphocytes in the peritoneal cavity of $gp91^{phox-/-}$ mice at the equivalent time points of resolution in wild types. The activation of A_{2A} was certainly anti-inflammatory in $gp91^{phox-/-}$ mice by virtue of it inhibition of PMN numbers. However, when administered therapeutically at the equivalent phase of resolution in wild types, it did not bring about resolution in $gp91^{phox-/-}$ as defined by its inability to trigger lymphocyte repopulation. Along these lines, activating A_{2A} with CGS 21680 at an earlier time point which exerted classic anti-inflammatory effects as defined by reduced PMN numbers (Figure 5.4C and 5.5A-B) was also without effect on lymphocyte numbers. This suggests that other factors, besides that which signal cAMP are responsible for lymphocyte repopulation and reversion to homeostasis.

The finding of adenosine being secreted and cAMP expressed during the early onset phase of the zymosan-induce peritonitis was not surprising given the established role these factors play in counter-regulating innate-immune mediated tissue damage (Cronstein 1994; Sitkovsky and Lukashev 2005). The reappearance of cAMP and adenosine again at resolution, however, is a

reflection of our growing understanding of resolution being an active, immuno-suppressive event controlled by endogenous counter-regulatory stop signals.

From data presented in this study, A_{2A} is shown to be anti-inflammatory whilst not affecting pro-resolution pathways. However, it is well known that caffeine is a non-specific A_{2A} receptor antagonist as it can antagonise A_1 as well as A_{2A} but possesses a lower affinity for the A_3 receptor (Fredholm, Battig et al. 1999). Resulting from its non-specific inhibition of A_{2A} caffeine may therefore worsen inflammation and negatively affect pro-resolution pathways. Indeed, dosing animals with 3 mg/kg caffeine or its stable analogue just before zymosan injection exaggerated the inflammatory response 4 h later and also impaired resolution. This is important as the amount of caffeine administered to the mice was equivalent to a realistic 1-2 cups of coffee. Increasing doses of caffeine to unrealistic 10-30 mg/kg, as was also done in this current study, caused a loss of caffeine's pro-inflammatory impact as at these levels and higher (100 mg/kg) caffeine may become a PDE4 inhibitor resulting in cAMP elevation, as shown *in vivo* (Ohta, Lukashev et al. 2007). Given the wide consumption of caffeine in the form of coffee and tea at least, we need to be aware of the data presented here and by others (Ohta, Lukashev et al. 2007) which emphasised that interfering with endogenous protective pathways such as adenosine at realistic levels of socially-consumed beverages will hamper innate immune responses. However, any attempts to increase caffeine intake in the hope of inhibiting PDE4 in order to dampen inflammation *via* cAMP elevation would require prohibitively high quantities of the drug. Thus, the most likely result of social caffeine consumption would be pro-inflammatory and resolution toxicity. Whether this leads to impaired ability to combat infections concomitant with prolonging resolution require further investigation.

In conclusion, when the endogenous anti-inflammatory pathways were examined in CGD, both adenosine and its intracellular signalling molecule, cAMP, show dysregulation. The reduced

levels of adenosine and downstream cAMP synthesis in a murine model of CGD lead to a state of pro-inflammation and PMN trafficking with no apparent attempts at resolution due to the persistence of the inflammatory stimulus. Rescuing this hyper-inflammatory state with A_{2A} agonists shows powerful anti-inflammation that does not bring about resolution as it inhibits PMN trafficking but does not initiate lymphocyte repopulation and reversal to homeostasis. Nonetheless it does suggest a potential treatment regime to dampen the hyper-innate immune component of CGD-associated infections.

Summary

Inflammation is fundamentally a beneficial response leading to removal of the offending factor. Resolution of inflammation is an active regulated process that is essential to maintain tissue integrity and function. Zymosan peritonitis, a resolving model of innate inflammation was an ideal platform to study the cellular profile during acute inflammation and the mediators that enable its resolution. In the naïve state, the peritoneal cavity consists of resident lymphocytes and macrophages. During acute inflammation, PMN influx peaked at 6-12 hours following zymosan, concomitant with a drop in lymphocyte numbers during this period. Resolution is signalled by the influx of phagocytosing macrophages and re-population with lymphocytes.

Previous work in our department showed a role for COX-2 derived PGD_2 and its cyclopentenone metabolite 15d-PGJ_2 during resolution of acute inflammation in a pleurisy model. Hematopoietic prostaglandin D_2 synthase ($hPGD_2S$) metabolises cyclooxygenase (COX)-derived PGH_2 to PGD_2 and 15-deoxy$\Delta^{12\text{-}14}$ PGJ_2 (15d-PGJ_2). Unlike COX, the role of $hPGD_2S$ in host defence remains ambiguous. For instance, PGD_2 can be either pro- or anti-inflammatory depending on disease aetiology while the existence of 15d-PGJ_2 and its relevance to pathophysiology remains highly controversial. In chapter 3, studies on $hPGD_2S$ knockout mice reveal that 15d-PGJ_2 is indeed synthesised in self-resolving peritonitis, detected using LC-MS-MS. As metabolism of PGD_2 to 15d-PGJ_2 can occur ex-vivo, by spiking inflammatory fluids *in situ* with deuterated PGD_2, the detected 15d-PGJ_2 was proven to be native and not deuterated 15d-PGJ_2. PGD_2 working on its DP1 receptor controls the balance of pro- versus anti-inflammatory cytokines that regulate leukocyte influx as well as monocyte-derived macrophage efflux from the inflamed peritoneal cavity to draining lymph nodes leading to resolution. Specifically, inflammation in $hPGD_2S$ knockouts is more severe during the onset phase arising from a substantial cytokine imbalance resulting in enhanced PMN and monocyte trafficking. Moreover, resolution is impaired being characterised by macrophage and surprisingly lymphocyte accumulation. Hence, data from this study places $hPGD_2S$ at the centre of controlling the onset as well as the resolution of acute

inflammation where it acts as a crucial checkpoint controller of cytokine/chemokine synthesis as well as leukocyte influx and efflux. In doing so we provide definitive proof that 15d-PGJ_2 is synthesised during mammalian inflammatory responses and highlight DP1 receptor activation as a potential novel anti-inflammatory strategy.

As mentioned above, lymphocyte numbers vary during the resolving innate inflammation and despite sub-populations of lymphocytes possessing innate immune-regulatory properties, seldom are their role in acute inflammation and its resolution discussed. In chapter 4, PGD_2 may have a role in the disappearance of lymphocytes in acute inflammation. In lymphocyte-deficient $RAG2^{-/-}$ mice, peritoneal T/B lymphocytes control PMN trafficking by regulating cytokine synthesis. Once inflammation ensues in normal mice, lymphocytes disappear in response to DP1 receptor activation by prostaglandin D_2. However, upon resolution lymphocytes repopulate the cavity comprising B1, NK, gamma/delta T, $CD4^+/CD25^+$ and B2 cells. Repopulating lymphocytes does not appear to have a role in resolution as inflammation in $RAG2^{-/-}$ and wild types resolve uniformly. However, repopulating lymphocytes are critical for modulating responses to super-infection. In $gp91phox^{-/-}$ mice, an animal model of chronic granulomatous disease, a genetic condition of impaired phagocytosis, not only is resolution delayed compared to wild types but there is a failure of lymphocyte reappearance. This predisposes to an exaggerated immune responses upon secondary challenge that is rescued by resolution-phase lymphocytes. The transition in T/B cells from acute inflammation to resolution has a central role in modulating the severity of early onset and orchestrating responses to secondary infection. As lymphocyte repopulation is also evident in human peritonitis, the phenotype of resolution phase lymphocytes and its function in modulating recurrent infection require further investigation.

In chronic granulomatous disease (CGD) there is failure to generate reactive oxygen metabolites resulting in recurrent infections and persistent inflammatory events. Therefore, $gp91phox^{-/-}$ mice

is an ideal platform to investigate critical mediators that lead to failure of resolution and chronic inflammation (Chapter 5). As responses to sterile stimuli in murine models of CGD also result in non-resolving inflammation, we investigated whether defects in endogenous counter-regulatory mechanisms and/or pro-resolution pathways contribute to the aetiology of CGD. To this end I carried out a series of experiments that demonstrated, in the first instance that adenosine and cAMP, which dampen innate immune-mediated responses, show a biphasic profile in resolving peritonitis; peaking at onset, waning as inflammation progresses and rising again at resolution. In resolving human peritonitis there was also elevations in adenosine and cAMP. In gp91phox$^{-/-}$ mice, an experimental model of CGD, levels of adenosine and cAMP were significantly lower at onset and again at resolution. Corroborating the finding of others, we show that adenosine, signalling through its A_{2A} receptor and therefore elevating cAMP is not only anti-inflammatory but, importantly, it does not impair pro-resolution pathways, properties typical of nonsteroidal anti-inflammatory drugs. Conversely, antagonising the A_{2A} receptor worsens acute inflammation and prolongs resolution. Taking this further, activating the A_{2A} receptor in gp91phox$^{-/-}$ mice was dramatically anti-inflammatory regardless of the phase of the inflammatory response A_{2A} agonists were administered i.e. onset or resolution demonstrating wide and robust pharmacological flexibility that is unlikely to subvert pro-resolution pathways.

This thesis therefore demonstrates that just like acute inflammation, resolution is an active process with a critical role PGD_2 and 15d-PGJ_2 in enabling this both by governing the influx and efflux of cells involved in the inflammatory process. Failure of this does lead to chronic inflammation as demonstrated by CGD mice, where the intracellular levels of adenosine and downstream cAMP are significantly reduced. The interplay between cyclo-oxygenase pathway and adenosine require further investigation.

Limitations of the study

Collection of samples from patients who develop peritonitis

One of the goals of the study was to determine the role of PGD_2 and 15d-PGJ_2 in patients on peritoneal dialysis who develop peritonitis, the most common complication of this modality. However there was variability in the timing of collection of the peritoneal effluent compared to the onset of symptoms. Patients in all cases developed peritonitis whilst at home. They then drain out the bag and bring it over to the hospital. Firstly this could happen any time during the day. There was a significant time lag between onset and collection of samples and during that period it is not stored in a controlled temperature leading to degradation of mediators of inflammation and resolution. This may explain why both PGD_2 and 15d-PGJ_2 were not detected in human peritoneal effluents.

The role of PGD_2 and 15d-PGJ_2 in human peritonitis

In the context of human peritonitis, the role of PGD_2 and 15d-PGJ_2 is not known. Furthermore there is a genetic heterogeneity in the expression of PGD_2 synthase expression in human and this is not known in the patients studied. For instance, in Japanese children, a single nucleotide polymorphism in the second intron of the hPGD_2 synthase gene located in 4q22.3 predicted a risk of asthma (Noguchi, Shibasaki et al. 2002).

In the murine model, I was able to demonstrate the role of PGD_2 in efflux of macrophages during resolution of peritoneal inflammation. This is an attractive area to investigate in humans but there were no techniques available to do so.

Role of re-populating lymphocytes in resolution of inflammation

In chapter 4, I was able to show that repopulating lymphocytes did not enable resolution but was protective against a secondary insult based on experiments in lymphocyte deplete $Rag1^{-/-}$ mice. Adoptive transfer of resolution phase lymphocytes into the peritoneal cavity of $gp91^{phox}$ knockouts was protective against a second insult. However repopulating lymphocytes comprise of B1 cells, NK and gamma/delta T cells as well as $CD4^{+}/CD25^{+}$ cells. It was not clear which sub-population was directly responsible or whether all members of the resolution phase lymphocytes was necessary for this beneficial action.

Future work

1. Investigation of genetic polymorphism of PGD2 synthase expression in patients with chronic kidney disease

This will allow an exploration of mutations to the PGD_2 synthase gene and risk of developing recurrent infections including peritonitis

2. Use LC-MS-MS to determine 15d-PGJ2 levels of inpatients on peritoneal dialysis that develop peritonitis.

This would make it easier to collect and store samples in a timely manner to minimize degradation of mediators of inflammation. This will allow to compare the lipid mediator profile in both resolving and non-resolving peritonitis

3. Pilot clinical studies with PGD2 agonists in peritoneal inflammation

4. Compare lymphocytes function from resolving and non-resolving peritonitis by ex-vivo culture.

When patients present with peritonitis and are admitted, the dialysis effluent is removed every 6 hours in patients on continuous ambulatory peritoneal dialysis. Therefore discreet cell populations can be harvested from the effluent and cultured ex-vivo. If the peritonitis fails to resolve, the catheter is surgically removed at day five days following onset. Therefore cellular samples can be obtained for the first five days of the acute peritonitis episode.

5. Investigating mediators responsible for resolution of inflammation in patients on dialysis.

Based on measurement of inflammatory markers (eg: highly sensitive c-reactive protein) dialysis patients (either on haemo or peritoneal) are significantly inflamed compared to individuals without chronic kidney disease. It is likely that there is an imbalance between the pro and anti inflammatory pathways in arachidonic acid metabolism (both COX and lipoxygenase). Using a cantharidin-induced skin blisters, we can investigate non inflamed versus chronically inflamed patients on dialysis and compare with non inflamed healthy volunteers for cellular as well lipid mediator profile (PGD_2, 15d-PGJ_2 and LXA_4)

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Hematopoietic prostaglandin D_2 synthase controls the onset and resolution of acute inflammation through PGD_2 and 15-deoxyΔ^{12-14} PGJ_2

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Hematopoietic prostaglandin D_2 synthase ($hPGD_2S$) metabolizes cyclooxygenase (COX)-derived PGH_2 to PGD_2 and 15-deoxyΔ^{12-14} PGJ_2 (15d-PGJ_2). Unlike COX, the role of $hPGD_2S$ in host defense is ambiguous. PGD_2 can be either pro- or antiinflammatory depending on disease etiology, whereas the existence of 15d-PGJ_2 and its relevance to pathophysiology remain controversial. Herein, studies on $hPGD_2S$ KO mice reveal that 15d-PGJ_2 is synthesized in a self-resolving peritonitis, detected by using liquid chromatography–tandem MS. Together with PGD_2 working on its DP1 receptor, 15d-PGJ_2 controls the balance of pro- vs. antiinflammatory cytokines that regulate leukocyte influx and monocyte-derived macrophage efflux from the inflamed peritoneal cavity to draining lymph nodes leading to resolution. Specifically, inflammation in $hPGD_2S$ KOs is more severe during the onset phase arising from a substantial cytokine imbalance resulting in enhanced polymorphonuclear leukocyte and monocyte trafficking. Moreover, resolution is impaired, characterized by macrophage and surprisingly lymphocyte accumulation. Data from this work place $hPGD_2S$ at the center of controlling the onset and the resolution of acute inflammation where it acts as a crucial checkpoint controller of cytokine/chemokine synthesis as well as leukocyte influx and efflux. Here, we provide definitive proof that 15d-PGJ_2 is synthesized during mammalian inflammatory responses, and we highlight DP1 receptor activation as a potential antiinflammatory strategy.

antiinflammatory | cyclooxygnease | drug development | eicosanoids | innate immunity

Cyclooxygenase (COX) metabolizes phospholipase A_2-derived arachidonic acid to prostaglandin (PG)H_2, which is further metabolized by a series of downsteam synthases to the prostanoids. Indeed, the expression of the particular downstream enzyme and its coupling either to constitutively expressed COX1 or to inducible COX2 will determine the profile and levels of arachidonic metabolites released by cells. Thus, targeting COX will diminish most if not all prostanoids, which, in the case of new-generation COX2 inhibitors, resulted in prostacyclin abatement, enhanced risk of cardiovascular side effects, and the eventual withdrawal of selective COX2 inhibitors from clinical usage. In this event, attention has now shifted to understanding the role of COX downstream synthases in inflammation and the cardiovascular system in the hope of adding more selectivity with fewer side effects.

In an attempt to understand the role of COX-related downstream synthase in host defense, we found that the COX2/hematopoietic PGD_2 synthase pathway resolves both acute innate (1, 2) and adaptive immune responses (3). Hematopoietic PGD_2 synthase ($hPGD_2S$) metabolizes COX-derived PGH_2 to PGD_2 (4), which may activate two G protein-coupled receptors, DP1 and DP2. DP1 regulates dendritic cell function (5), and DP2 promotes allergic inflammation (6–8). Although controversial, it is believed that PGD_2 is initially converted to PGJ_2 and 15-deoxy-PGD_2 (15d-PGD2) in an albumin-independent manner. Thereafter, the cyclopentenone PGs (cyPGs) 15-deoxy-$\Delta^{12,\ 14}$-PGJ_2 (15d-PGJ_2) and Δ^{12}-PGJ_2 are generated from PGJ_2 by albumin-independent and -dependent reactions, respectively (9). Although 15d-PGJ_2 is a putative ligand for peroxisome proliferator-activated receptor (PPAR)γ (10), the skepticism with cyPGs lies in whether 15d-PGJ_2 is formed in pathophysiological settings at sufficient levels to exert meaningful biological effects in mammalian systems.

Investigating the role of $hPGD_2S$ in acute inflammation, we provide definitive proof using liquid chromatography–tandem MS (LC-MS/MS) that 15d-PGJ_2 is synthesized *in vivo*. In a series of mechanistic studies, we show that along with PGD_2 acting on its DP1 receptor, 15d-PGJ_2 controls the balance of cytokines and chemokines that regulate leukocyte trafficking during acute inflammation as well as the efflux of macrophage to draining lymphatics leading to its resolution such that in $hPGD_2S$-deficient mice, inflammation is grossly exaggerated and fails to resolve. These data not only prove that cyPGs exist in mammalian systems and exert potent antiinflammatory and proresolution properties, but data from this work also highlight the potential of activating DP1 receptors as a future antiinflammatory strategy.

Results

$hPGD_2S$-Derived Lipid Mediators in Acute Inflammation. In the first instance, levels of PGD_2 and 15d-PGJ_2 were quantified throughout the time course of resolving murine peritonitis. PGD_2 was maximal 2 h after zymosan injection, with levels waning by 24 h (Fig. 1*A*). Contrary to common belief, 15d-PGJ_2 was detectable in cell-free inflammatory exudates of wild-type mice by LC-MS/MS (Fig. 1 *B* and *C*). It was elevated at 2 h, maximum between 6 and 24 h, and had declined by 48 h/72 h, with average levels of between 0.5 and 5 ng/ml. PGD_2 and 15d-PGJ_2 were absent from experimental WT controls (0 h). Given its instability (9), we controlled for potential *ex vivo*

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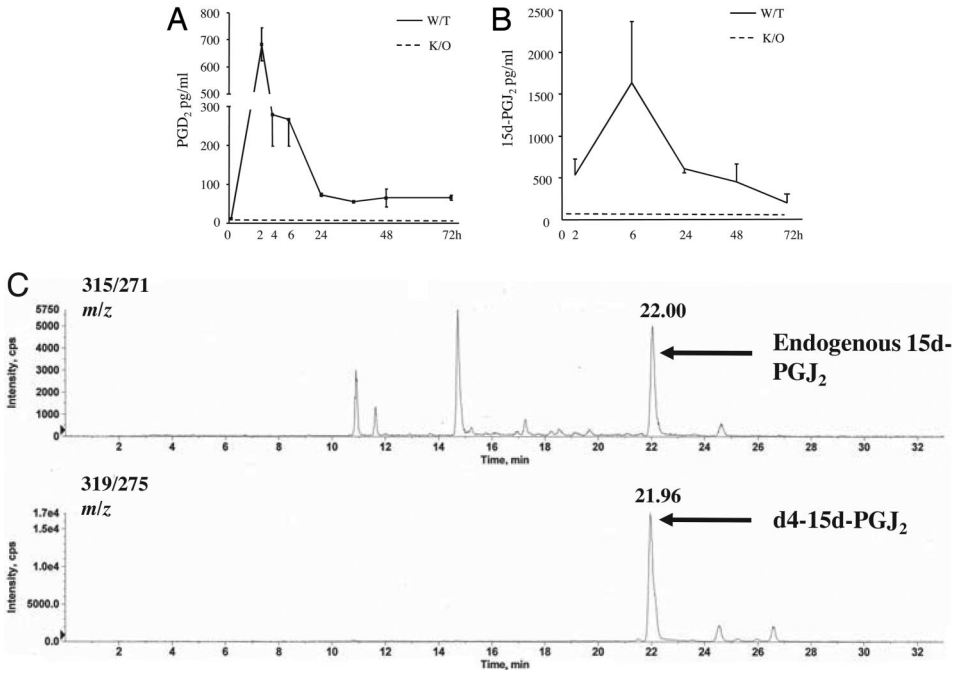

Fig. 1. hPGD$_2$S synthesizes 15d-PGJ$_2$ during zymosan-induced resolving peritonitis. (*A* and *B*) hPGD$_2$S-derived PGD$_2$ (*A*) and 15d-PGJ$_2$ (*B*) were extracted from the cell-free inflammatory exudates and quantified by EIA and electrospray triple quadruple LC-MS/MS, respectively. *Ex vivo* degradation of PGD$_2$ to 15d-PGJ$_2$ during sample processing was controlled for by spiking inflammatory fluids *in situ* with deuterated PGD$_2$. (*C*) LC-MS/MS spectra of detected 15d-PGJ$_2$, which was found to be native and not deuterated. For eicosanoid analysis $n = 6–8$ animals were present in each group with the exception of 15d-PGJ$_2$, which had 2–6 samples per group. Data are represented as the mean ± SEM.

degradation of PGD$_2$ to 15d-PGJ$_2$ during sample processing by spiking inflammatory fluids *in situ* with deuterated PGD$_2$ and found that the detected 15d-PGJ$_2$ was native and not deuterated 15d-PGJ$_2$. These data, coupled with the finding that neither PGD$_2$ nor 15d-PGJ$_2$ was detectable in the exudates of hPGD$_2$S KOs (Fig. 1 *A* and *B*), confirmed that 15d-PGJ$_2$ is a bona fide PGD$_2$ eicosanoid metabolite formed *in vivo* during resolving inflammation.

hPGD$_2$S Tempers the Severity of Acute Inflammation Through DP1 and 15d-PGJ$_2$. We sought to determine the role that PGD$_2$ and 15d-PGJ$_2$ play in inflammation. hPGD$_2$S$^{-/-}$ mice show a more aggressive early (4–6 h) response to zymosan, characterized by a 2-fold increase in polymorphonuclear leukocyte (PMN) influx compared with WT (Fig. 2*A*). By 48 h, PMN numbers in KOs were reduced to levels similar to that found in WT (Fig. 2*A*, *Inset*), indicating that although hPGD$_2$S controls early PMN influx it does not influence their survival or clearance from inflammatory sites. This was confirmed by detecting little difference in PMN apoptosis by annexin V/propidium iodide (PI) labeling as well as total leukocyte apoptosis by the same technique and by caspase-3 activity [supporting information (SI) Fig. 5]. Increased inflammation in hPGD$_2$S$^{-/-}$ mice at onset was associated with a decrease in antiinflammatory IL-10 (Fig. 2*B*) and an increase in proinflammatory TNFα (Fig. 2*C*) and monocyte chemoattractant protein 1 (MCP-1) (Fig. 2*D*). This hyperinflammatory phenotype in KOs was rescued to that of WT at 6 h by a selective DP1 (BW245C, Fig. 2*E*) but not a DP2 receptor agonist [15(*R*)-15-methyl PGD$_2$] (Fig. 2*F*), independently of apoptosis. Treatment of hPGD$_2$S$^{-/-}$ mice with BW245C also restored the imbalance in TNFα and MCP-1 vs. IL-10 to levels found in WT (Fig. 2 *G–I*). Thus, by signaling through the DP1 receptor, hPGD$_2$S-derived PGD$_2$ controls the onset phase of acute inflammation and its balance of pro- and antiinflammatory cytokines and chemokines. A range of other cytokines and chemokines were measured, and their relative levels in wild types and hPGD$_2$S KOs are presented in SI Table 1. 15d-PGJ$_2$ also reduced cell numbers in the peritoneal cavity of mice but by increasing leukocyte apoptosis as determined by both annexin V/PI labeling and caspase activity, which resulted in a concomitant elevation in IL-10 and TGFβ1, cytokines typically released by macrophage upon recognition of apoptotic leukocytes (SI Fig. 6 *A–F*). We believe that these data do not represent the true role of 15d-PGJ$_2$ *in vivo* because there is little difference in total cell apoptosis between WT and KOs at onset (5.05 ± 0.6% of controls expressing phosphatidylserine vs. 3.9 ± 0.64% for KOs). These potentially misleading data may arise from injecting a bolus dose of exogenous and highly reactive electrophilic 15d-PGJ$_2$ vs. its controlled endogenous release by intracellular hPGD$_2$S. To understand how endogenous 15d-PGJ$_2$ works *in situ* and to bypass the apoptosis-inducing effects caused by a single bolus injection, we examined its effects on peritoneal leukocytes *ex vivo* below.

hPGD$_2$S Exerts Its Protective Effects on Resident Macrophages and Lymphocytes. In the naïve or uninflamed murine peritoneum, T and B lymphocytes constitute ≈40% of the total cell population (≈1.5 × 10^6), with the remaining being resident macrophages, determined by using CD3/CD19, B220, CD5, and F4/80 labeling quantified by FACS. In the case of the peritoneal cavity, resident macrophages dictate the magnitude of the ensuing response with peritoneal lymphocytes possessing innate immune regulatory effects (11–13). In this event, we next determined on which cell types within the peritoneum PGD$_2$ and 15d-PGJ$_2$ exert their antiinflammatory effects. Separated peritoneal T and B cells as well as macrophages were stimulated with either LPS or zymosan (B cells and macrophages) or anti-CD3 antibody (T cells). Cytokines were measured 24 h later in response to BW245C (DP1 receptor agonist), 15(*R*)-15-methyl PGD$_2$ (DP2 receptor agonist), or 15d-PGJ$_2$ at concentrations that do not increase apoptosis (SI Fig. 7). IL-10 from hPGD$_2$S-deficient peritoneal T cells was significantly lower than WT but was reversed by BW245C (DP1 agonist); neither 15(*R*)-15-methyl PGD$_2$ (DP2 receptor agonist) nor 15d-PGJ$_2$ had any effect (Fig. 3*A*). Similarly, IL-10 secretion from zymosan or LPS-stimulated hPGD$_2$S-deficient peritoneal B cells (265 ± 20 and 178 ± 12 pg/ml, respectively) was lower compared with WT [387 ± 25 ($P \leq 0.05$) and 625 ± 35 ($P \leq 0.01$) pg/ml, respectively] and was rescued by BW245C only (Fig. 3 *B* and *C*). Conversely, synthesis of TNFα from these zymosan- or LPS-stimulated hPGD$_2$S-deficient B cells (1,625 ± 175 and 1,260 ± 152 pg/ml, respectively) was higher compared with WT [1,160 ± 105 ($P \leq 0.05$) and 810 ± 65 pg/ml ($P \leq 0.05$), respectively] but were reduced by BW245C and 15d-PGJ$_2$ but not with 15(*R*)-15-methyl PGD$_2$ (Fig. 3 *D* and *E*). In contrast to T and B lymphocytes, IL-10 from peritoneal macro-

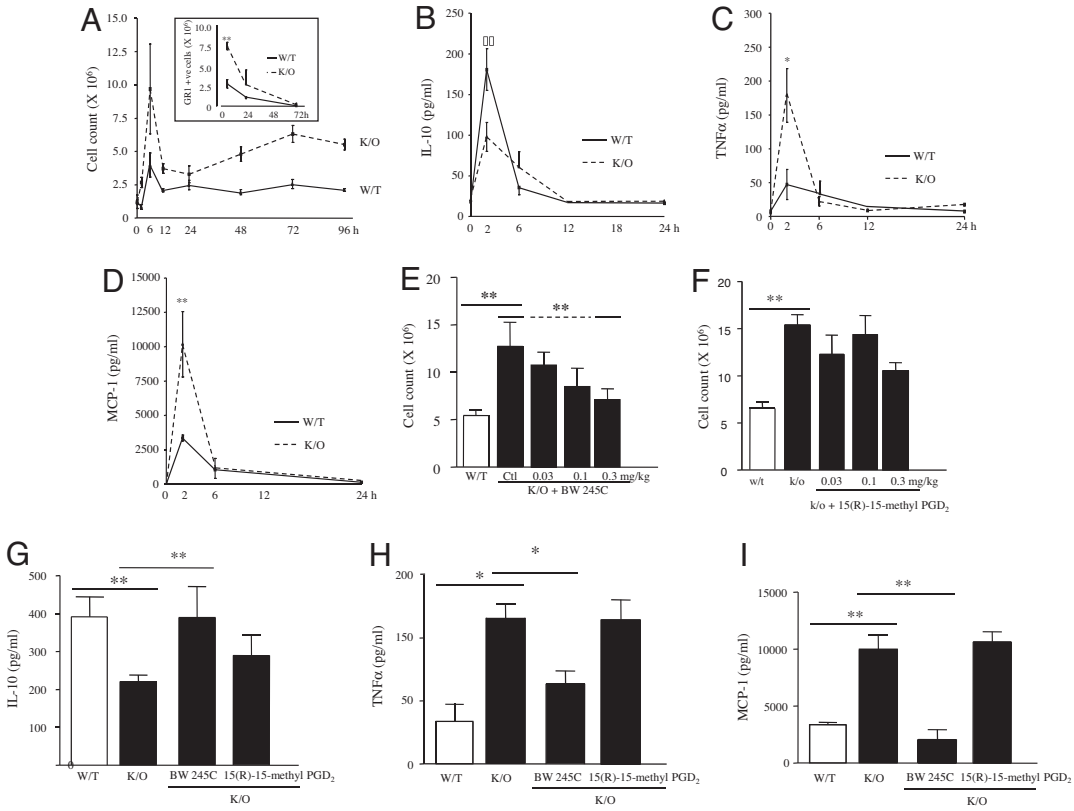

Fig. 2. PGD_2 acting by the DP1 receptor controls the balance of pro- vs. antiinflammatory mediators and leukocyte trafficking during acute inflammation. (*A*) Intraperitoneal zymosan resulted in a self-limiting inflammatory response that peaked at 6–12 h. Inflammation in $hPGD_2S^{-/-}$ mice (K/O) was 2-fold greater than in WT at 6 h and failed to resolve, with the early-onset phase characterized by predominantly GR1-positive PMNs with higher numbers of monocytes/macrophages compared with WT (see Fig. 4*A*). (*Inset*) Appropriate isotype control antibodies. (*B–D*) Cell-free exudate levels of IL-10 (*B*) were lower in $hPGD_2S^{-/-}$ mice whereas TNFα (*C*) and MCP-1 (*D*) were higher. (*E–I*) The hyperinflammation in $hPGD_2S^{-/-}$ mice was redressed by the DP1 receptor agonist BW245C (*E*) but not by the DP2 agonist 15(*R*)-15-methyl PGD_2 (*F*), resulting in a corresponding (*G–I*) equilibration of inflammatory cytokines and chemokines to levels similar to controls. $n = 6–8$ animals per group; *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA followed by Bonferroni *t* test, with data expressed as mean ± SEM.

phages was not elevated by BW245C (Fig. 3*F*), DP2 receptor activation, or by 15d-PGJ_2 (data not shown). However, these cells did show a reduction in TNFα and MCP-1 after treatment with BW245C and 15d-PGJ_2, respectively (Fig. 3 *G* and *H*). Similar results were obtained by using bone marrow-derived macrophages from these animals. These data show that $hPGD_2S$-derived lipid mediators exert differentially protective effects on peritoneal-resident macrophages as well as lymphocytes, thereby controlling the balance of cytokines and chemokines that orchestrate innate inflammatory responses.

$hPGD_2S$ Deficiency Severely Compromises Resolution. Finally, one striking finding of these studies was impaired resolution in $hPGD_2S^{-/-}$ mice characterized by macrophage (Fig. 4*A*) as well as lymphocyte accumulation ($0.24 \pm 0.04 \times 10^6$ T and B cells in WT vs. $0.8 \pm 0.1 \times 10^6$ in KOs; $P \leq 0.01$). SI Fig. 8 shows a breakdown of lymphocyte subtypes in these animals. The implication of lymphocyte accumulation during resolution in $hPGD_2S^{-/-}$ mice is unknown at this stage but is reminiscent of results published showing the persistence of lymphocytes in the inflamed paws of COX2 KO mice (14) and raises questions regarding the long-term impact of COX inhibitors on the progression of chronic inflammatory diseases. Although increased macrophage numbers during resolution in $hPGD_2S^{-/-}$ mice may have arisen from elevated MCP-1 (Fig. 2*D*) and macrophage inflammatory protein 1β (MIP-1β levels) (Fig. 4*B*), a failure to clear to the draining lymphatics (15, 16) may also provide an explanation. To investigate this hypothesis, the selective macrophage label PKH26-PCL (15, 16) was injected i.p. at the peak of macrophage accumulation (24 h), revealing increased numbers of PKH26-PCL-labeled macrophages in the peritoneal cavities of $hPGD_2S^{-/-}$ mice (Fig. 4*C*) and a corresponding reduction in the parathymic load of these cells compared with WT at 72 h (Fig. 4*D*). Adding BW245C or 15d-PGJ_2 to $hPGD_2S^{-/-}$ mice alleviated peritoneal macrophage accumulation (Fig. 4*E*) and increased numbers of PKH26-PCL macrophages in the parathymic lymph nodes (Fig. 4*F*). The DP2 receptor agonist was without effect. Notably, BW245C can be added 6 h, 12 h, or 24 h after inflammation and will exert a macrophage clearance effect at all of these time points, attributing a robust proresolution property to DP1 (data not shown). Thus, $hPGD_2S$-derived PGD_2 and 15d-PGJ_2 play a role in clearing inflammatory macrophages from the inflamed peritoneum to local draining lymph nodes.

Discussion

We describe a central role for $hPGD_2S$ in controlling the onset and resolution of innate immune-mediated inflammation. First, we show that the protective nature of $hPGD_2S$ is mediated by DP1 and 15d-PGJ_2, which control the balance of pro- and antiinflammatory cytokine synthesis. These data contrast with the pathogenic role PGD_2 plays in allergic inflammation through DP2 (6–8). However, as with the entire field of eicosanoid biology, lipid mediators can be either protective or pathogenic depending on disease etiology, cell types present, and eicosanoid receptor expression (17). For instance, signaling through DP2 causes bronchoconstriction as well as Th2 and eosinophil cell accumulation (6–8). In contrast, PGD_2 activation of DP1 may also suppress asthmatic symptoms by tar-

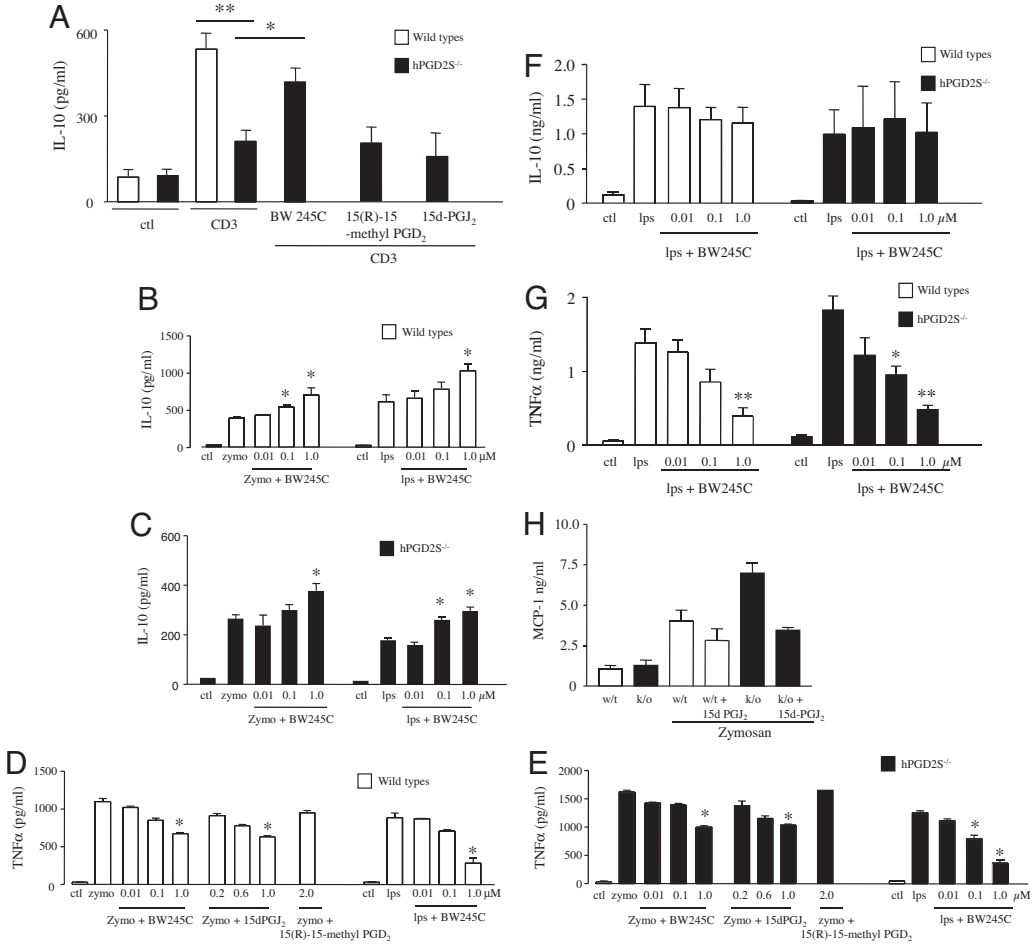

Fig. 3. hPGD$_2$S controls the inflammatory phenotype of peritoneal leukocytes. (*A–C*) IL-10 release from stimulated hPGD$_2$S-deficient T cells (*A*) as well as B cells (*B* and *C*) was not only lower than that from wild types but was rescued by DP1 activation (BW245C). DP2 receptor activation with 15(*R*)-15-methyl PGD$_2$ was without effect in these experiments. (*D* and *E*) Conversely, TNFα release from hPGD$_2$S-deficient B cells was greater than that from wild types and was reversed with BW245C and 15d-PGJ$_2$ but not 15(*R*)-15-methyl PGD$_2$. (*F–H*) In macrophages, whereas hPGD$_2$S had little effect on IL-10 (*F*), BW245C reduced TNFα (*G*) whereas 15d-PGJ$_2$ also ameliorated levels of MCP-1 secretion (*H*). These data show the differential regulatory effects of hPGD$_2$S-derived lipid mediators on inflammatory leukocyte phenotype. Cells were isolated from $n = 3$ animals, and all experiments done in triplicate on two separate occasions to confirm original findings. *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as mean ± SEM.

geting lung dendritic cells, resulting in increased Treg cells that dampen inflammation in an IL-10-dependent manner (18). We found no difference in the numbers of CD4$^+$/CD25$^+$ cells between hPGD$_2$S$^{-/-}$ and WT in experimental peritonitis (SI Fig. 9), and we propose that in acute inflammation at least, the protective effects of PGD$_2$ are directed toward modulating resident leukocyte inflammatory phenotypes and are not reliant on recruitment of Treg cells. On this theme, PGD$_2$ along with PGE$_2$ can also induce PMN 15-lipoxygenase expression in a partly cAMP-dependent manner, causing the subsequent release of antiinflammatory and proresolution lipoxin A$_4$ (19). It is conceivable, therefore, that some of the protective and cytokine regulatory effects of PGD$_2$ in acute inflammation may also arise from its induction of other families of protective lipid mediators. Thus, from the current data and that published by others (5, 18), we suggest that DP1 receptor activation may be an attractive antiinflammatory target capable of modulating both innate and adaptive immune responses of different etiologies. In particular, we found that DP1 activation exerts its antiinflammatory effects at many stages throughout the inflammatory response, i.e., when administered either prophylactically or therapeutically, thereby demonstrating greater pharmacological flexibility than, for example NSAIDs, which although effective during acute stages of inflammation, subvert proresolution pathways (1, 19).

Controversy exists over whether 15d-PGJ$_2$ is synthesized *in vivo* and whether it possesses relevant patho/physiological functions. This debate arose, in part, when we originally reported that COX2-derived PGD$_2$ and 15-dPGJ$_2$ brought about acute inflammatory resolution (1). Controversy also arose not so much because we suggested that COX2 was protective at a time when the emphasis was on developing COX2 inhibitors, but because we used an ELISA to quantify 15d-PGJ$_2$. In particular, the reactive nature of 15d-PGJ$_2$ raised questions regarding the accuracy of using an antibody-based measuring system. This result, coupled with subsequent reports using physical methods to show only negligible levels of 15d-PGJ$_2$ in various biological systems (20), questioned the importance of 15d-PGJ$_2$ in biology. In this work we show, by using LC-MS/MS on samples obtained from a resolving inflammation, that 15d-PGJ$_2$ does exist *in vivo* at levels up to 5 ng/ml. We controlled for degradation of unstable PGD$_2$ to 15d-PGJ$_2$ during sample processing by spiking inflammatory fluids *in situ* with deuterated PGD$_2$, and we found that the detected 15d-PGJ$_2$ was native and not deuterated 15d-PGJ$_2$. These data, coupled with the finding that neither PGD$_2$ nor 15d-PGJ$_2$ was detectable in the exudates of hPGD$_2$S KOs (Fig. 1 *A* and *B*) and that the hyperproliferative phenotype of hPGD$_2$S$^{-/-}$ T cells from a delayed-type hypersensitivity reaction was reversed with 15d-PGJ$_2$ (3), confirm that 15d-PGJ$_2$ is an endogenously generated protective PGD$_2$ metabolite formed *in vivo* during resolving inflammatory reactions. The importance of this finding cannot be underestimated. cyPGs, derived from PGs of the A or D series possess antiinflammatory (21), antiviral (22), and anticancer (23) properties by activating either nuclear membrane-bound PPARs (24) or by forming covalent adducts with thiols through the unsaturated carbonyl group in the cyclopentenone moiety (25, 26). Importantly, protein modification by cyPGs does not occur randomly with cyPGs targeting defined cysteine residues within certain proteins in an apparently pH-dependent manner (27). Moreover, structural determinants of either the protein or the cyPG may be important for the specificity of protein modification such that cyPGs with diverse structures could selectively modify distinct proteins in cells. Thus, levels of

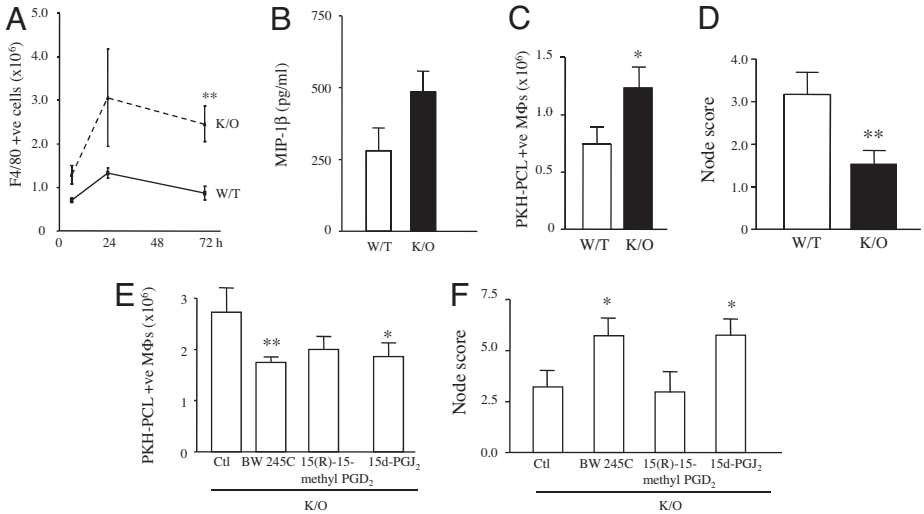

Fig. 4. $hPGD_2S$ deficiency compromises inflammatory resolution. (*A* and *B*) Although PMNs dominate the inflamed peritoneal cavity at the early-onset phase in $hPGD_2S^{-/-}$ (K/O) mice (Fig. 2*A*, *Inset*) F4/80 positive macrophages were the predominant cell types during resolution (*A*) WT, wild type. Increased macrophage numbers in $hPGD_2S^{-/-}$ mice could arise from enhanced MIP-1β (*B*) as well as MCP-1 (Fig. 2*D*) synthesis and/or a failure to exit the peritoneum to the parathymic lymph nodes (15, 16). To determine the latter, mice were injected i.p. with the macrophage label PKH-PCL26 at either 24 h or 48 h. (*C*) A significantly greater number of PKH26-PCL-positive macrophages (double labeling with FITC-labeled F4/80) were recorded in the cavity of $hPGD_2S^{-/-}$ mice compared with WT at 72 h. (*D*) Correspondingly fewer labeled macrophages were found by histology in the parathymic lymph nodes of the KOs. (*E* and *F*) Adding BW245C (DP1 receptor agonist) or 15d-PGJ_2 to the inflamed cavity of $hPGD2S^{-/-}$ mice reversed the accumulation of macrophages in the peritoneum (*E*) and resulted in increased numbers of labeled macrophages in parathymic lymph nodes (*F*). $n = 10$ animals per group; *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as mean ± SEM.

cyPGs in the extracellular environment may only represent the tip of the iceberg, and their true level in biological systems may be much higher. The lack of detectable cyPGs in biological fluids does not necessarily exclude their existence within cells and their potential to exert meaningful biological effects. Indeed, in a series of experiments we estimate that of 15d-PGJ_2 added exogenously to biological systems, >50% binds BSA. Moreover, in cardiomyocyte cell cultures, >80% binds to culture medium supplemented with 10% FCS with ≈16% binding to or being metabolized by cardiomyocytes, leaving <4% detectable by LC-MS/MS (SI Fig. 10). It is for this reason that we need to add quantitatively more 15d-PGJ_2 back to inflammatory models to mimic the biological effects of endogenously produced 15d-PGJ_2. This requirement is illustrated in SI Fig. 6, where more 15d-PGJ_2 is injected i.p. than is detectable in peritoneal fluids (Fig. 1*B*). We suggest that the role of 15d-PGJ_2 in self-limiting inflammatory responses is manifold and does not necessarily overlap with that of PGD_2, controlling chemokine and cytokine synthesis as well as intracellular signaling and leukocyte apoptosis. We found that exogenous 15d-PGJ_2 exerts proresolution effects if used pharmacologically by enhancing leukocyte apoptosis, the net outcome being similar to that found with the cyclin-dependent kinase inhibitor roscovitine, which enhanced PMN apoptosis and hastened resolution (28).

In addition to tissue resident histiocytes, there is the influx of monocytes during inflammation, which differentiate into large granular macrophages designed to phagocytose effete leukocytes. The fate of Reiter cells (macrophages containing dead cells) during resolution is poorly understood, with evidence that macrophages can undergo programmed cell death (29) and/or clearance from the peritoneum to the parathymic lymph nodes in a very late antigen (VLA)-4- and VLA-5-dependent manner (15, 16). Although the control of phagocyte clearance is poorly understood, we found an excess of macrophages in the inflamed peritoneum of $hPGD_2S^{-/-}$ mice and a corresponding deficit of these cells in the draining lymph nodes compared with controls. This result could have arisen from accelerated monocyte influx, mediated by elevated MCP-1 and MIP-1β (Figs. 2*D* and 3*B*) and/or failed lymphatic efflux. Using a phagocytosable fluorescent cell tracker, the peritoneal pool of accumulated macrophages in $hPGD_2S^{-/-}$ mice at resolution was found to be reduced by BW245C or 15d-PGJ_2 and was associated with an increase in macrophages in the parathymic lymph node. We therefore suggest that DP1 is a robust pharmacological target that, in addition to stemming PMN trafficking, could be used to disseminate macrophages from sites of chronic inflammation where macrophages play a pathogenic role. A similar result was found recently with ω-3 polyunsaturated fatty acid-derived resolvin E1 and protectin D1, which facilitated leukocyte trafficking to lymph nodes and spleen, collectively underscoring the proresolution properties of lipid mediators in acute inflammation (30).

Lymphocytes were also found to accumulate in $hPGD_2S^{-/-}$ mice at resolution, which we suspect resulted from enhanced influx in response to elevated chemokines in these animals rather than increased local lymphocyte proliferation because there was no significant difference in thymidine incorporation in $hPGD_2S^{-/-}$ lymphocytes compared with controls (data not shown). Indeed, there was a trend toward a reduction in lymphocytes from KOs in these experiments, in contrast to results obtained with $hPGD_2S$-deficient lymphocytes from an antigen-induced arthritis model (3), which showed elevated proliferation in $hPGD_2S$ KO mice. These differences are most likely the result of a differential role for both $hPGD_2S$-derived lipid mediators and lymphocyte subsets and activation states in inflammatory disease of dissimilar etiologies.

In summary, we provide proof that $hPGD_2S$ synthesizes 15d-PGJ_2 during mammalian defense responses and together with PGD_2, acting through the DP1 receptor, plays a central role in controlling the onset of acute inflammation and its resolution by controlling the balance of pro- vs. antiinflammatory cytokines as well as macrophage clearance through draining lymphatics. Here, we have highlighted the potentially antiinflammatory and proresolution properties of cyPGs as well as DP1 receptors.

Materials and Methods

Animal Maintenance and Induction of Inflammation. $hPGD_2S$ KO mice were generated as described (3). Animals were bred under standard conditions and maintained in a 12 h/12 h light/dark cycle at 22 ± 1°C and given food and tap water ad libitum in accordance with United Kingdom Home Office regulations. Peritonitis was induced by the i.p. injection of 1 mg of type A zymosan (Sigma) in 0.5 ml of sterile PBS. Cells were obtained by using 2 ml of sterile PBS to wash out the inflamed peritoneal cavity, and they were enumerated by hemocytometer at the time points stated in *Results*.

Eicosanoid Analysis. Samples stored at −20°C were thawed at room temperature and spiked with 4 ng of the internal standard d4–15d-PGJ_2 and acidified to pH 3. Solid-phase extraction was performed with Varian 3M Empore high-performance extraction disk cartridges, and the columns were washed with 1 ml of H_2O and 1 ml of heptane and eluted with 1 ml of ethyl acetate. The eluate was dried under nitrogen and analyzed by electrospray triple/quadruple LC-MS/MS (Sciex API 3000; PerkinElmer). The conditions for the LC-MS/MS were: C18 columns (100 × 0.2 mm) with elution volume 200 μl/min consisting of a mobile phase 0–1 min of distilled H_2O (pH 3), MeCN 75:25%,

followed by a gradient mobile phase to 100% MeCN. 15d-PGJ_2 was detected and quantified in negative ion mode, and the electrospray potential was maintained at −4 to 4.5 kV and heated to 500°C. For MS-MS analysis, 15d-PGJ_2/internal standards were subjected to collision-induced fragmentation. Tetradeuterated (d4) 15d-PGJ_2, PGD_2, and 15d-PGJ_2 were purchased from Cayman Chemicals. Varian 3M Empore high-performance extraction disk cartridges were purchased from JVA Analytical, Ltd. All other common laboratory chemicals were purchased from Sigma. For PGD_2, samples were extracted C18 columns, treated with methoxylamine hydrochloride (MOX HCl), and the resulting stable PGD_2-MOX was measured by EIA (Cayman Chemicals).

Cytokine/Chemokine, FACS, and Caspase Activity Analysis. Cytokines and chemokines were measured initially by multiplex cytokine array analysis (Bio-Rad) using the manufacturer's protocols. Specific mediators of interest [IL-10, TNFα (eBiosciences), and MCP-1 (Becton Dickinson)] were further quantified by ELISA according to manufacturer's instructions. FACS was carried out on Becton Dickinson FACSCalibur with data analyzed by Cellquest. Leukocytes were incubated with antibodies to CD3 (Serotec), B cells (Ly220; Serotec), natural killer and γ/δ T cells (gift from T. Hussell, Kennedy Institute, U.K.), GR1 (BD PharMingen), or F4/80 (Caltag Laboratories) using isotype antibodies (Serotec) and compensated as appropriate for dual labeling. For apoptosis, cells were incubated with annexin V/PI (Becton Dickinson). For caspase 3 activity, frozen cell pellets were lysed in ice-cold RIPA buffer [20 nM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% SDS Promega)] for 10 min. After centrifugation at 16,000 × *g* for 15 min at 4°C, supernatants were aspirated, and protein was quantified by the Bradford reaction. Samples were incubated with 50 μM substrate in caspase assay buffer [213.5 mM Hepes (pH 7.5), 31.25% (wt/vol) sucrose, and 0.3125% CHAPS] for 1 h, and fluorescence was measured on a microplate reader (Fluostar Galaxy; BMG Laboratory Technologies) with excitation at 380 nm and emission set at 460 nm. For each sample, four replicates were assayed, with two replicates containing 50 μM the caspase-3 inhibitor (Ac-DEVD-CHO) and the remaining pair containing vehicle. Fluorescence readings from wells containing inhibitor were subtracted from total fluorescence, and results were calculated as nmol of aminomethyl couramin (AMC) per mg of protein per min.

Leukocyte Separation and *in Vitro* Culturing. Bone marrow-derived or peritoneal macrophages were isolated by adherence to the bottom of 6-well tissue culture plates after incubation for 90 min at 37°C in a humid incubator. Nonadherent cells were removed and used to isolate T and B lymphocytes. The adherent cells were eluted with Versene, washed with 2% FCS in HBSS, and resuspended at 10^7 cells per ml. These cells were further depleted of contaminant T and B cells by using magnetic beads coated with rat monoclonal antibodies to mouse CD3 or B220 (Dynal Biotech, Ltd.). T and B lymphocytes were isolated by using the Dynal mouse B cell (or T cell) negative isolation kit according to the manufacturer's instructions. In brief, a mixture of rat monoclonal antibodies with specificity to all mouse non-B cells (or non-T cells when isolating T cells) was added to cell suspensions and incubated for 20 min at 4°C. Cells coated with the added monoclonal antibodies were then removed with magnetic beads coated with sheep polyclonal antibody to rat Ig. The purity of the cells was regularly >95%. B lymphocytes and macrophages were counted by hemocytometer and cultured in RPMI medium 1640 with 10% (vol/vol) FCS and antibiotics [100 units/ml benzylpencillin, 10 μg/ml streptomycin, 2.5 μg/ml amphotericin (all from Sigma–Aldrich)] in sterile 24-well culture plates (VWR) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were incubated with the selective DP1 agonist (BWC245; Cayman), DP2 agonist [15(*R*)-15 methyl PGD_2; Cayman), or 15d-PGJ_2 (Cayman) with medium as control and then stimulated with 100 μg/ml zymosan type A (Sigma), 1 μg/ml LPS, or vehicle. The experiment was terminated 24 h later, and supernatant was stored at −80°C until further use. The protocol for T cells was similar apart from stimulation with anti-CD3 antibody.

Macrophage Clearance Assays. Two milliliters of 500 nM macrophage-specific stain PKH26-PCL (Sigma) was injected into the inflamed peritoneal cavity with cells and parathymic lymph nodes isolated at time points stated in results section. Cells staining positive for PKH26-PCL (FL2 channel) were identified with FITC-labeled F4/80 (FL1 channel). Staining was found almost exclusively within macrophages. Parathymic lymph nodes were extracted and snap frozen in OCT1, and serial sections (minimum 15 sections per node) were examined for the presence of fluorescent PKH26-PCL-labeled macrophages. All sections were scored blindly by two independent observers by using an Olympus Axioscop microscope and our validated analog scale (16).

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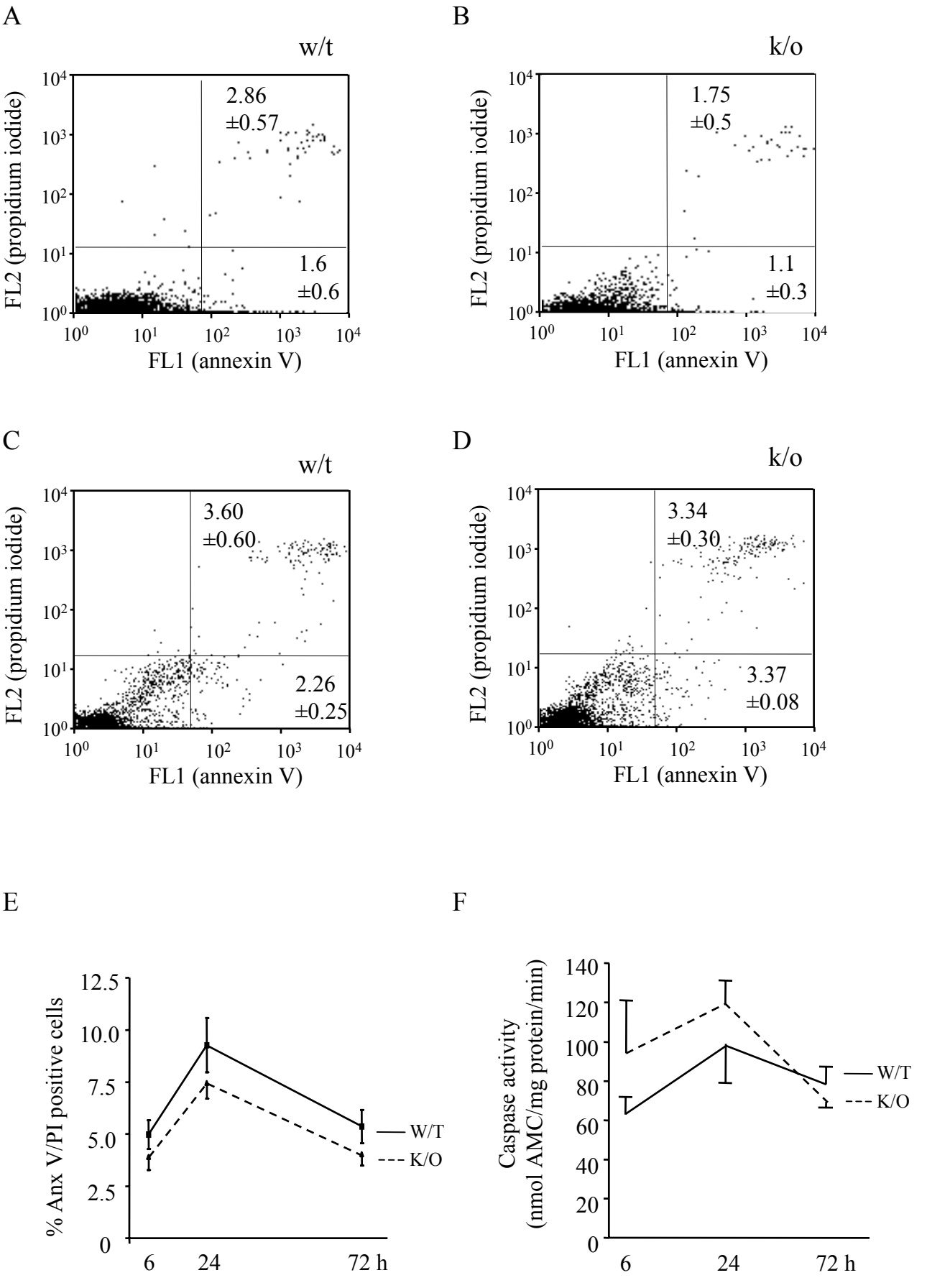
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±0.57
1.6
±0.6
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FL1 (annexin V)
B
k/o
1.75
±0.5
1.1
±0.3
C
w/t
3.60
±0.60
2.26
±0.25
D
k/o
3.34
±0.30
3.37
±0.08
E
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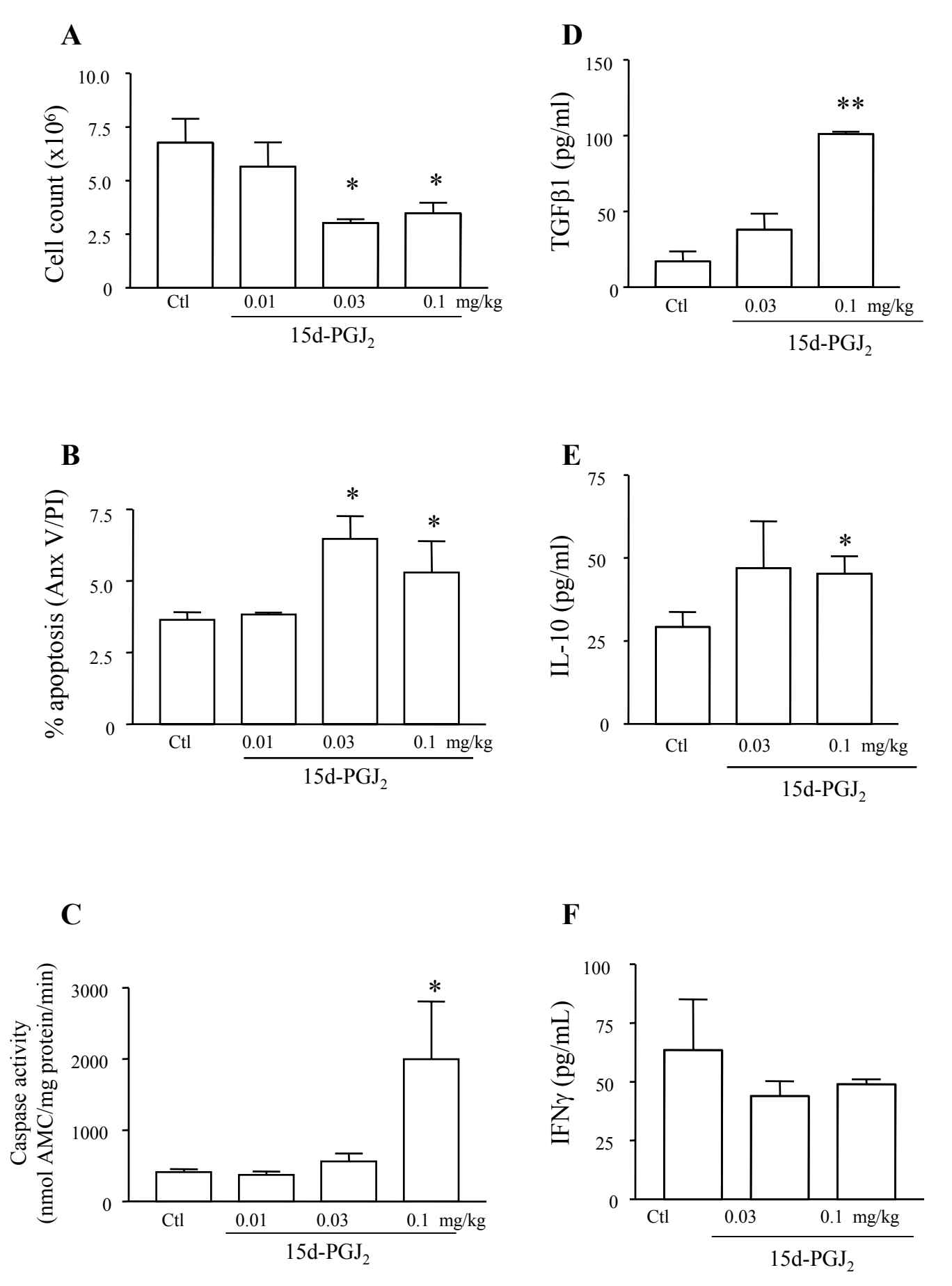
W/T
K/O
6
24
72 h
F
Caspase activity
(nmol AMC/mg protein/min)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5.0
2.5
0
Ctl
0.01
0.03
0.1 mg/kg
15d-PGJ2
B
% apoptosis (Anx V/PI)
7.5
5.0
2.5
0
Ctl
0.01
0.03
0.1 mg/kg
15d-PGJ2
C
Caspase activity
(nmol AMC/mg protein/min)
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2000
1000
0
Ctl
0.01
0.03
0.1 mg/kg
15d-PGJ2
D
TGFβ1 (pg/ml)
150
100
50
0
Ctl
0.03
0.1 mg/kg
15d-PGJ2
E
IL-10 (pg/ml)
75
50
25
0
Ctl
0.03
0.1 mg/kg
15d-PGJ2
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0.03
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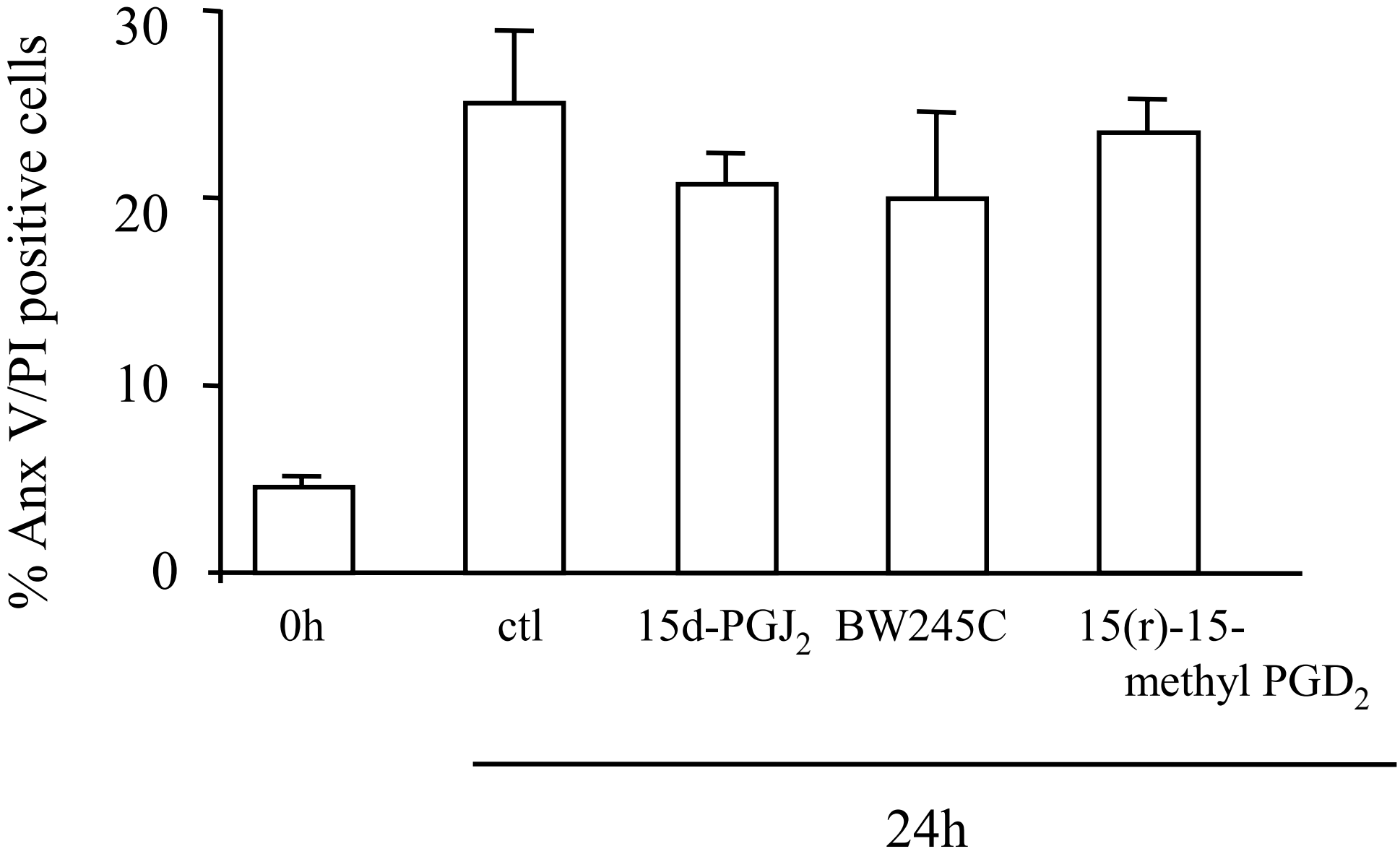
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BW245C
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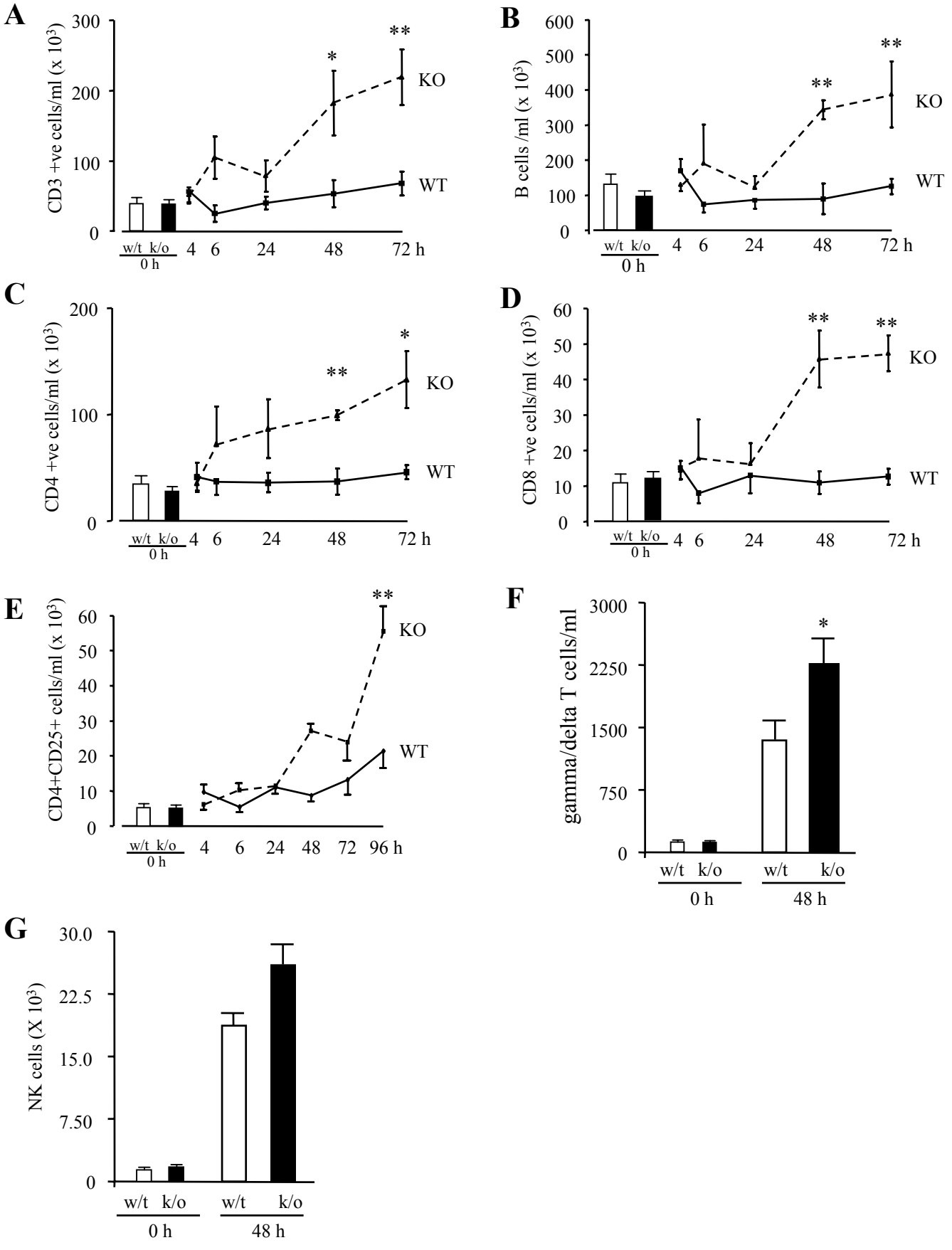
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200
100
0
*
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WT
w/t k/o
0 h
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B
B cells /ml (x 10³)
600
500
400
300
200
100
0
**
**
KO
WT
w/t k/o
0 h
4 6 24 48 72 h
C
CD4 +ve cells/ml (x 10³)
200
100
0
**
*
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WT
w/t k/o
0 h
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D
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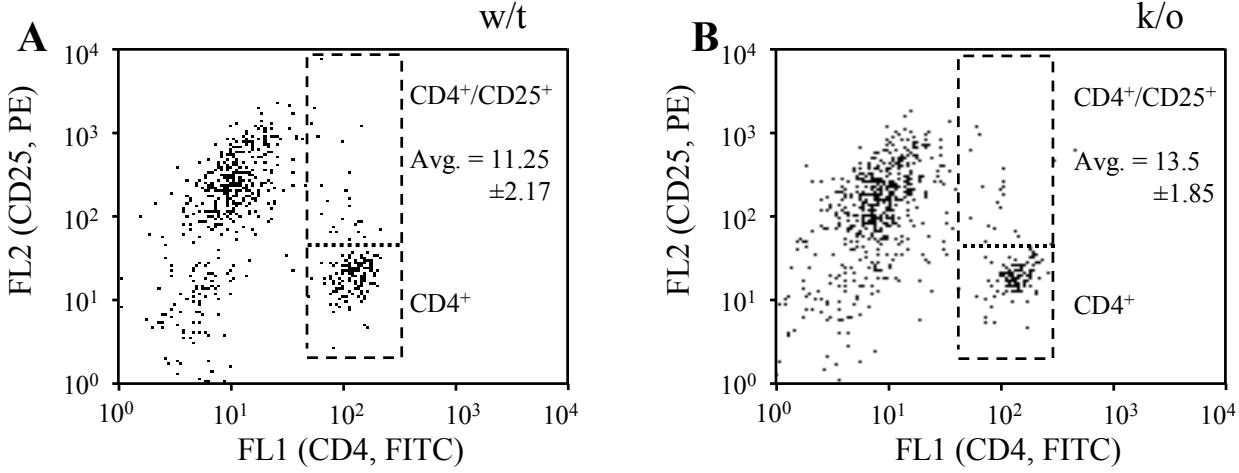
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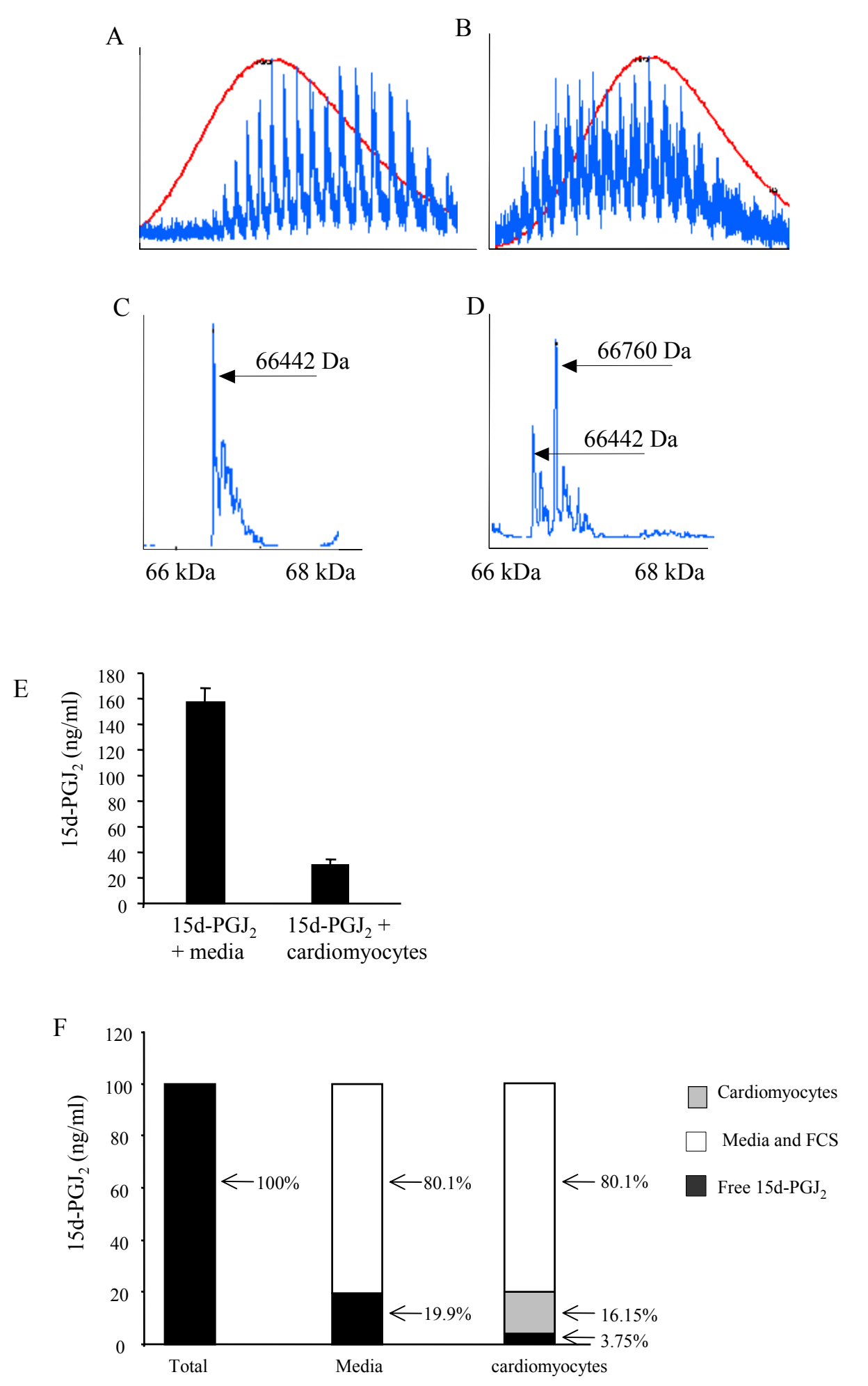
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160
140
120
100
80
60
40
20
0
15d-PGJ2 + media
15d-PGJ2 + cardiomyocytes
F
15d-PGJ2 (ng/ml)
120
100
80
60
40
20
0
100%
80.1%
19.9%
80.1%
16.15%
3.75%
Total
Media
cardiomyocytes
Cardiomyocytes
Media and FCS
Free 15d-PGJ2

Resolving the problem of persistence in the switch from acute to chronic inflammation

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The rapid induction of an inflammatory response is crucial for effective host defense. A wide range of proinflammatory agents ensure that this response occurs. Prominent among these are members of the prostaglandin and leukotriene family that are enzymatically derived from arachidonic acid (AA, C20:4) (1). At the onset of inflammation, AA is metabolized via either cyclooxygenases (COX) to produce prostaglandins (PGs) and thromboxanes or lipoxygenases to generate leukotrienes (LTs) (1). These ecicosanoids induce and help establish the inflammatory infiltrate. However, at some point during the inflammatory cascade, inhibition and ultimately resolution of the inflammatory response must occur; otherwise, tissue homeostasis cannot occur and inflammation persists.

Until relatively recently, the resolution of inflammation was thought to occur mainly as a result of the passive withdrawal of proinflammatory mediators. It is now clear that the inflammatory program contains key checkpoints as well as temporal and spatial switches that regulate its onset, maintenance, and resolution (2). A key finding in recent years is that the biosynthetic pathways required for inflammation to resolve often require the prior production of lipid mediators produced during the induction phase of inflammation. In particular, PGE_2 and PGD_2, which are responsible for inflammation induction, subsequently stimulate antiinflammatory circuits by inducing the lipoxin-producing enzyme 15-lipoxygenase in neutrophils. This class switch in eicosanoid production, from LTs and PGs to lipoxins (3), provides temporal and spatial checkpoints during the inflammatory cascade (see Fig. 1). In this issue of PNAS, Rajakariar *et al.* (4) provide yet another twist in the resolution story with the demonstration that class switching may not always be required for resolution to be initiated. They provide compelling evidence that, within a single eicosanoid family pathway initiated by COX-2 and subsequently regulated by the enzyme hematopoietic PGD_2 synthase ($hPGD_2S$), proresolving mediators PGD_2 and 15-deoxyΔ^{12-14} PGJ_2 (15d-PGJ_2) are produced at sufficient levels *in vivo* to drive the resolution of acute inflammation.

The concept that antagonists that limit the extent of a biological cascade are generated as the cascade develops is familiar in other self-limiting pathways such the complement and coagulation cascades. However, homeostasis takes an unexpected twist in inflammation. Events at the onset of acute inflammation establish biosynthetic circuits for a series of chemical mediators that later serve not only as antagonists but also as agonists for an opposing program: resolution. These proresolution agonists don't just inhibit the cascade, they actively dismantle it. Therefore, antiinflammation and proresolution are not equivalent. The agonists that actively promote resolution (an emerging family of proresolving lipid mediators, including lipoxins, resolvins, and protectins) are inherently different from the antagonists that limit the extent of the inflammatory response, at both the molecular and the cellular level (2).

Ever since the discovery that aspirin exerts many of its antiinflammatory actions through inhibition of the COX enzymes (5), there has been keen interest in developing specific inhibitors of these enzymes. However, the logic for such an approach did not account for the fact that the inducible COX-2 enzyme is also responsible for the production of antiinflammatory eicosanoids, some of which are important in maintaining vascular homeostasis (6). In a provocative paper published in 1999, Gilroy *et al.* (7) suggested that COX-2 may have antiinflammatory properties. Subsequent findings by Fukunaga *et al.* (8) and Schwab *et al.* (9) have since demonstrated that blocking prostaglandin biosynthesis with COX-2 inhibitors, although effective at the very early stages of the inflammatory cascade, delays the onset of resolution (8) and/or impairs phagocyte removal of dead cells (9). In effect, these potent COX-2 selective agents lead to friendly fire by being "resolution toxic."

In a series of seminal papers, Gilroy and colleagues have built on their original observations to present a convincing case that some of the downstream products from the COX-2-derived PGD_2 family, and in particular 15d-PGJ_2, are important in the resolution of acute inflammatory responses (9, 10). Critics of these studies have pointed out that a molecular mechanism that detailed how PGD_2 leads to resolution was missing. More damning were the comments that the antibody-based methods used to measure these metabolites, and the negligible levels of 15d-PGJ_2 found in various biological systems, made the biological importance of their original observations questionable (11). Using both genetic and biochemical studies, Rajakariar *et al.* (4) now provide compelling evidence, complete with a molecular mechanism that should silence the critics.

$hPGD_2S$ metabolizes COX-derived PGH_2 to PGD_2. However, the role of $hPGD_2S$ in host defense remains unclear because it and its metabolites appear to display both pro- and antiinflammatory properties, depending on the disease model and the way in which they are administered (12). This ambiguous role for $hPGD_2S$ is even more problematic because PGD_2 is further metabolized nonenzymatically to produce PGs of the J series, including PGJ_2 and 15d-PGJ_2, whose relevance to pathophysiology has remained highly controversial. In an attempt to clarify the role of $hPGD_2S$ in inflammation, Rajakariar *et al.* (4) used mice deficient in the enzyme to unequivocally demonstrate that $hPGD_2S$ is responsible for the production of all PGD_2 and 15d-PGJ_2 during zymosan-induced resolving peritonitis. Using liquid chromatography–tandem MS (LC-MS/MS), they provide physical evidence for *in vivo* formation of 15d-PGJ_2 in resolving exudates in a peritonitis model of inflammation at levels up to 5 ng/ml (≈15 nM). They noticed that macrophage emigration from the inflamed tissues to draining lymph nodes was delayed in $hPGD_2S$ knockout mice. This delay was rescued by addition of DP1-specific agonists, emphasizing the important role of DP1 in controlling both the onset of acute inflammation and its resolution. Surprisingly, lymphocyte numbers

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See companion article on page 20979.

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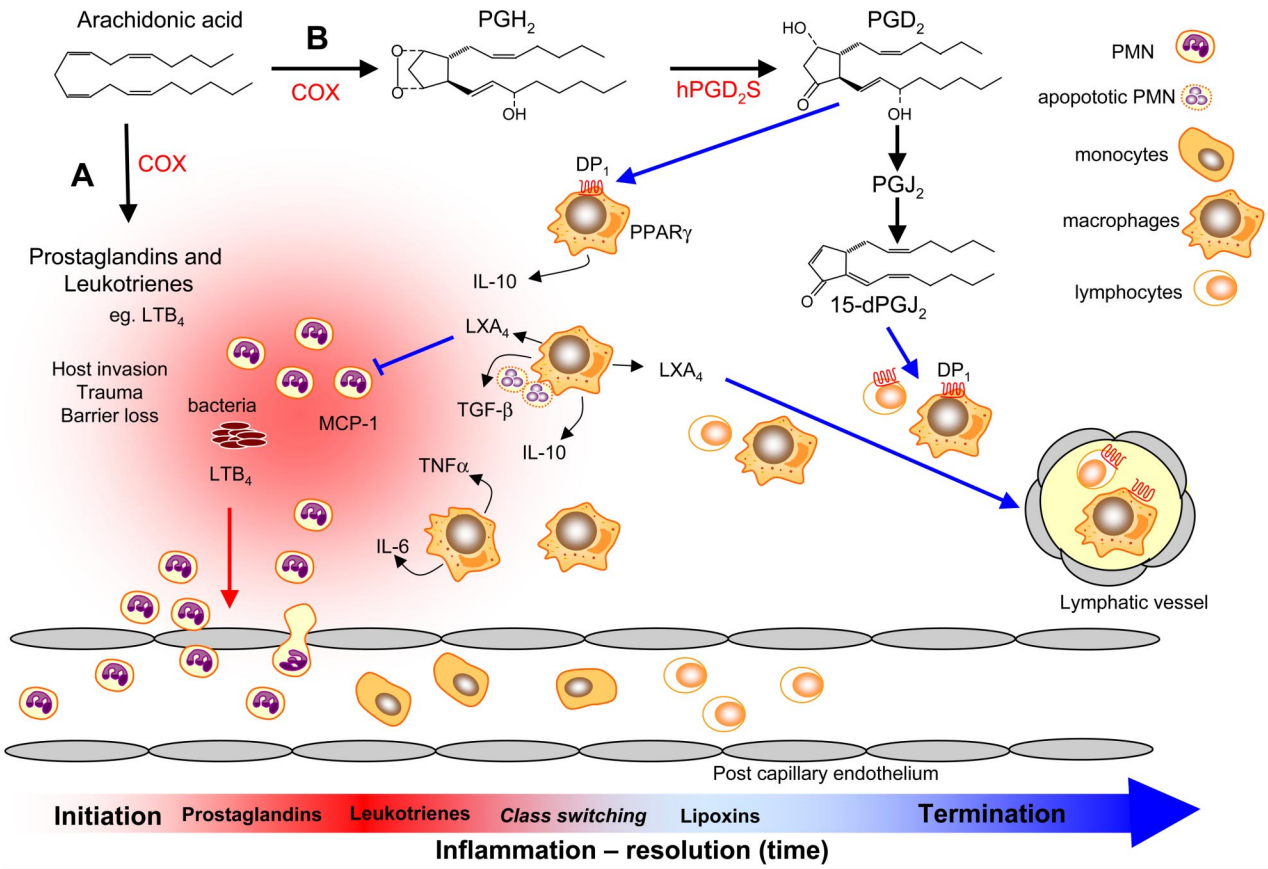

Fig. 1. Temporal and spatial regulation of resolution. (*A*) Current paradigm: Breaches in host defense such as pathogen invasion, host trauma, or loss of barrier function initiate the inflammatory response with AA rapidly converted to PGs and LTs (e.g., LTB_4) via COX enzymes. LTB_4 is a potent chemoattractant of neutrophils to the site of inflammation (red arrow). As the inflammatory response progresses, a temporal switch from initiation to resolution occurs with the transcellular generation of lipoxins (LXA_4) that inhibit the further recruitment of polymorphonuclear neutrophils (PMNs) (blue line). As the inflammatory response continues, monocytes are recruited via the release of the monocyte chemotactic protein-1. Macrophages release proinflammatory cytokines IL-6 and TNFα, but engulfment of apoptotic PMNs causes macrophages to change to a nonphlogistic phenotype releasing TGF-β, IL-10, and lipoxin. At the end of an acute inflammatory response, macrophages and lymphocytes leave the site of inflammation via the draining lymphatics, stimulated by lipoxins (blue arrow). (*B*) New addition to the paradigm: At the onset of the inflammatory response, PGD_2 is generated from AA via COX to PGH_2 and then converted to PGD_2 by $hPGD_2S$. PGD_2 is metabolized nonenzymatically to 15-$dPGJ_2$. PGD_2 acts on the DP1 receptor expressed on macrophages and lymphocytes, whereas 15-$dPGJ_2$ is likely to interact with peroxisome proliferator-activated receptor-γ. Working in tandem, they decrease proinflammatory cytokines such as TNFα and increase antiinflammatory cytokines such as IL-10, but like the lipoxin family, they also promote egress of macrophages and lymphocytes to the draining lymphatics (blue arrows).

increased in the $hPGD_2S$ knockout mice at a time when resolution was at its peak in wild-type mice. Although the authors were unable to pinpoint why this increase occurred, they suggest that it was not due to changes in proliferation or cell death. Clearly these issues will need to be addressed in the future, particularly as pharmacological intervention with exogenous 15d-PGJ_2 leads to accelerated leukocyte apoptosis, but at doses that are substantially higher than those formed naturally in the peritoneal exudates (13).

Although these results will excite those who work in the field of inflammation biology, and in particular its pathobiology and molecular pharmacology, they are of broad interest to investigators in many other fields, including host defense and cancer. For example, cyclopentone isoprostanes derived from PGs of the A and D series have been shown to possess antiviral and anticancer properties working through the activation of nuclear-membrane-bound peroxisome proliferator-activated receptors (12). However, the most exiting implication of these new findings is that they will encourage a shift in focus from the development of even more antiinflammatory, and potentially "resolution-toxic" drugs, toward "resolution-friendly" replacement therapies (14). Perhaps only then will the next generation of aspirin- and steroid-like compounds with the ability to both inhibit the induction of inflammation and jump-start its resolution emerge. It would appear that in the field of inflammation resolution, Cinderella has finally arrived at the ball.

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Novel biphasic role for lymphocytes revealed during resolving inflammation

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Novel biphasic role for lymphocytes revealed during resolving inflammation

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Acute inflammation is traditionally described as the influx of polymorphonuclear leukocytes (PMNs) followed by monocyte-derived macrophages, leading to resolution. This is a classic view, and despite subpopulations of lymphocytes possessing innate immune-regulatory properties, seldom is their role in acute inflammation and its resolution discussed. To redress this we show, using lymphocyte-deficient RAG1$^{-/-}$ mice, that peritoneal T/B lymphocytes control PMN trafficking by regulating cytokine synthesis. Once inflammation ensues in normal mice, lymphocytes disappear in response to DP1 receptor activation by prostaglandin D_2. However, upon resolution, lymphocytes repopulate the cavity comprising B1, natural killer (NK), γ/δ T, CD4$^+$/CD25$^+$, and B2 cells. Repopulating lymphocytes are dispensable for resolution, as inflammation in RAG1$^{-/-}$ and wild-type mice resolve uniformly. However, repopulating lymphocytes are critical for modulating responses to superinfection. Thus, in chronic granulomatous disease using gp91phox$^{-/-}$ mice, not only is resolution delayed compared with wild-type, but there is a failure of lymphocyte reappearance predisposing to exaggerated immune responses upon secondary challenge that is rescued by resolution-phase lymphocytes. In conclusion, as lymphocyte repopulation is also evident in human peritonitis, we hereby describe a transition in T/B cells from acute inflammation to resolution, with a central role in modulating the severity of early onset and orchestrating responses to secondary infection. (Blood. 2008;111:4184-4192)

Introduction

Inflammation is controlled by a balance of proinflammatory and antiinflammatory signals, resulting in the development of an immune response followed by temporally released proresolution factors that lead to inflammation switching off and injured tissues returning to normal physiology.[1] In this setting, attention is traditionally placed on phagocytes such as polymorphonuclear leukocytes (PMNs) and monocyte-derived macrophages, with comparatively less weight place on the importance of lymphocytes in acute inflammation and its resolution. Given their roles in early defense to bacteria and viruses, innate-type lymphocytes including B1 cells merit further exploration for their potential roles in host defense and restorative physiology, as it is becoming clear that diminished innate lymphocyte function or enhanced lymphocyte death by apoptosis, for instance, has being postulated to play a central role in the pathogenesis of burn injury[2,3] and sepsis,[4,5] respectively.

One of the major impetuses for this current investigation stemmed from our previous observations[6] and those made by others[7-10] showing lymphocytes repopulating sites of tissue injury as inflammation abates, suggesting that lymphocytes might help to switch off acute inflammation. Investigating this possibility in experimental peritonitis, T and B lymphocytes, normal residents of the naive peritoneum, were found to regulate the severity of the early onset phase of acute inflammation by elaborating anti-inflammatory cytokines and dampening PMN influx. However, once PMNs begin to accumulate, resident lymphocytes disappear in response to PGD_2 working through its DP1 receptor. As inflammation resolves, a unique profile of lymphocytes begin to repopulate the cavity. Repopulating lymphocytes, however, do not bring about resolution but replenish resolving tissues with the necessary cellular players (CD3, B1 cells, natural killer [NK] and γ/δ T cells as well as CD4$^+$/CD25$^+$ T cells) to control future innate immune-mediated responses. We provide relevance of these findings to human health by showing the absence of repopulating lymphocytes in nonresolving inflammation, which predisposes to secondary infection, resulting in severe inflammatory responses. Although lymphocytes in adaptive immunity are well understood, their role in innate immunity and resolution is highlighted here, as is their functional control by lipid mediators, of which there is a growing body of evidence. For instance, in addition to PGD_2,[11] lipoxins and aspirin-triggered epi-lipoxins inhibit human T-cell TNFα secretion,[12] while docosahexanoic acid–derived protectin D1 blocks T-cell migration and TNFα and IFNγ secretion and promotes apoptosis in human T cells.[13] Thus, from these current studies and those published by others,[12,13] we highlight the regulatory role played by n-3 and n-6 polyunsaturated fatty acid metabolites on lymphocyte function during acute inflammation and its resolution.

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Methods

Animal maintenance, induction of inflammation human peritonitis sampling

Hematopoietic prostaglandin D_2 (PGD_2) synthase ($hPGD_2S$) knockout mice were generated as previously described.[14] All other animals were bred under standard conditions and maintained in a 12-hour/12-hour light/dark cycle at 22 (±1)°C and given food and tap water ad libitum in accordance with United Kingdom Home Office regulations. Peritonitis was induced by the intraperitoneal injection of either type A zymosan (1 mg for all experiments unless otherwise stated), group B streptococcus (GBS), or LPS (1 mg/kg; Sigma-Aldrich, St Louis, MO), and cells were enumerated by haemocytometer at time points stated in "Results" by sterile phosphate-buffered saline (PBS) washout. Ethical approval (P/03/136A) for collection of human peritonitis samples was obtained from St Bartholomew's and the Royal London Hospitals from patients with end-stage renal failure undergoing peritoneal dialysis. For pharmacologic rescue experiments, BW245C (DP1 agonist[15,16]) or 15(R)-15-methyl PGD_2 (DP2 agonist[17]) in 100 μL of PBS (pH 7.2)/BSA 0.1% was injected at equal doses at 30 minutes prior to and after the zymosan.

Trypsinization of peritoneal cavity

To determining the fate of peritoneal T and B cells, cavities of mice bearing a 4-hour zymosan-induced peritonitis was lavaged with sterile PBS to remove accumulated inflammatory cells and edema. A total of 5 mL prewarmed 5% trypsin was then added to the peritoneal cavity for 10 minutes, followed by an equal volume complete medium to acquire cells adhered to the peritoneal lining/greater omental lymphoid organ. Cells were then analyzed for composition by fluorescence-activated cell sorter (FACS).

FACS analysis and cytokine/chemokine analysis

Cytokines were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, San Diego, CA). FACS was carried out on a Becton Dickinson Facscalibur with data analyzed by CellQuest (BD, Franklin Lakes, NJ). Leukocytes were incubated with antibodies for 30 minutes to either CD3/CD19 (Serotec, Oxford, United Kingdom), B cells (Ly220; Serotec), CD5 (BD PharMingen, San Diego, CA), MAC-1/CD11b (BD PharMingen), NK and γ/δ cells (gift from Dr T. Hussell, Kennedy Institute, London, United Kingdom), GR1 (BD PharMingen), or F4/80 (Caltag Laboratories, Burlingame, CA) using respective isotype antibodies as controls (Serotec) and compensated as appropriate for dual labeling. For apoptosis, cells were incubated with annexin V/propidium iodide (BD PharMingen) and analyzed on Becton Dickinson Facscalibur with data analyzed by CellQuest.

Leukocyte separation and analysis

Contents of resolving-phase peritoneal cavities of wild-type animals were isolated, and macrophages were separated from remaining lymphocytes by adherence to the bottom of 6-well tissue-culture plates. Nonadherent cells were removed and used to isolate T and B cells as well as NK and γ/δ T cells for transfer back in to $gp91^{phox}$ knockout mice using FACS and relevant antibodies to confirm that their composition and ratios reflects that present in situ at resolution. Resolving-phase lymphocytes were enriched at a concentration of 10^6/mL and 0.5 mL injected into $gp91^{phox}$ knockout mice. In addition, one of the problems with identifying discrete populations of cells such as those found at sites of inflammation by FACS from a larger mixed cell population is that the fluorescence of one cell type after labeling with a fluorescent antibody may be masked by the natural fluorescence of others. In order to confirm the cell types identified using cell-surface antigen markers, peritoneal lymphocytes and macrophages were also isolated and put back into the FACS with their forward- and side-scatter signatures compared against specifically labeled cells. Thus, after lymphocytes were removed from 6-well plates, adherent cells, mainly macrophages, were eluted with Versene, washed with 2% fetal calf serum (FCS) in Hanks balanced salt solution (HBSS) and resuspended in Dulbecco modified Eagle medium (DMEM). These cells were further depleted of contaminant T and B cells using magnetic beads coated with rat monoclonal antibodies to mouse CD3 or B220 (Dynal Biotech, Paisley, United Kingdom). T and B lymphocytes were isolated using the Dynal mouse B-cell (or T-cell) negative isolation kit according to the manufacturer's instructions (Dynal Biotech). In brief, a mixture of rat monoclonal antibodies with specificity to all mouse non-B cells (or non-T cells when isolating T cells) cells were added to cell suspensions and incubated for 20 minutes at 4°C. Cells coated with the added monoclonal antibodies were then removed with magnetic beads coated with sheep polyclonal antibody to rat Ig. Purity of the cells were regularly greater than 95%.

Bacterial culturing

The clinical GBS isolate, NCTC10/84 (serotype V) was grown in Todd Hewitt Broth (THB) without agitation at 37°C to an OD_{600} of 0.4, equivalent to 10^8 cfu/mL. Bacteria collected by centrifugation were washed with sterile PBS. Mice were inoculated intraperitoneally with 3×10^7 cfu NCTC in 30 μL PBS. For survival experiments, mice were inoculated by intraperitoneal injection with 5×10^7 cfu NCTC in 0.3 mL PBS.

Results

Biphasic trafficking of lymphocytes during acute inflammation

We[6] and others[7-10] have previously observed lymphocytes repopulating sites of injury as inflammation resolves, but without attributing a functional relevance to their reappearance. Trafficking of lymphocytes during resolution is apparent in inflammation associated with human chronic ambulatory peritoneal dialysis where, as inflammation decreases (Figure 1A,B), lymphocyte numbers increase (Figure 1C). Clinical assessment of these patients was based on patients presenting with abdominal pain, cloudy dialysate, and leukocyte count of more than 100/mm^3. In all cases, peritonitis resolved by day 5 as determined by the appearance of a clear dialysate and abatement of abdominal symptoms. Similar results were evident in resolving experimental murine peritonitis (Figure 1D). Experiments were therefore carried out to establish the role these cells play in innate immune-mediated inflammation by characterizing, in the first instance, the profile of lymphocyte populations throughout the time course of an acute zymosan-induced peritonitis. In the naive murine peritoneum (0 hours), lymphocytes constitute about 50% of the total cell population, with the remaining being resident macrophages. Of the CD3 cells, $CD4^+$ and $CD8^+$ cells were found as well as lower numbers of $CD4^+/CD25^+$, γ/δ T cells, and NK cells (Figure 1E-J). However, the majority of lymphocytes in the naive cavity are B cells constituting about 70% to 80% of the total lymphocyte population labeling positively for CD19 as well as B220 (Figure 1K). Of these B cells, about 80% are $B220^{low}/CD5^+/MAC\text{-}1^{low}$, indicative of a B1 phenotype, with the remainder being $B220^{high}/MAC\text{-}1^-$ B2 cells. As inflammation initiates (1-4 hours), T and B cells disappear but repopulate the peritoneum again between 12 and 24 hours (Figure 1D). Notably, there were more $CD4^+/CD25^+$, γ/δ T cells, and NK cells found during resolution (Figure 1H-J) as well as B1 cells expressing higher levels of MAC-1 than in the naive state (Figure 1K), collectively referred to hereafter as resolution-phase lymphocytes. Figure S6 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) shows FACS analysis of lymphocyte cell-surface labeling. As inflammation peaks between 6 and 12 hours in this model and subsequently resolves (Figure 1D), it is argued that lymphocytes repopulate the

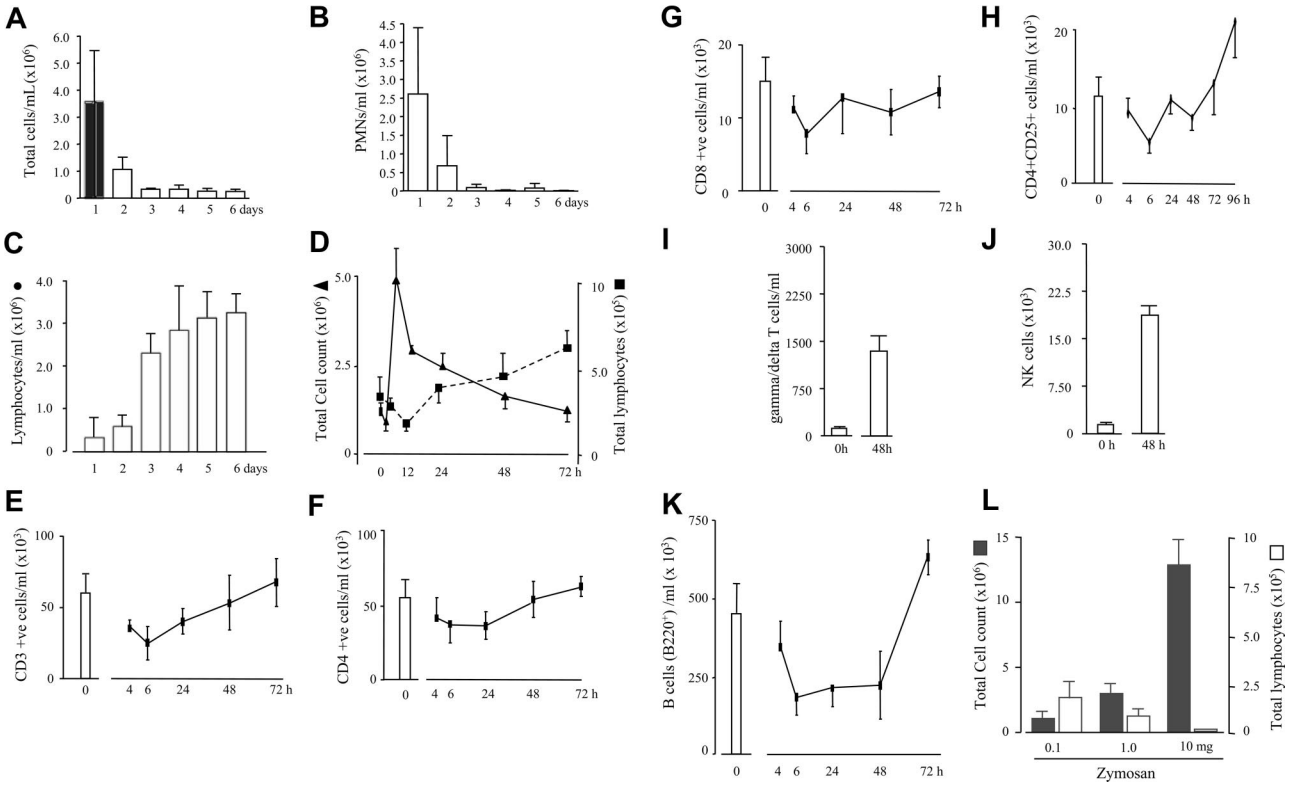

Figure 1. Biphasic trafficking of lymphocytes during acute inflammation. (A-C) Patients undergoing chronic ambulatory peritoneal dialysis and who developed peritonitis that resolved were found to have lymphocytes at the time of recovery of clinical symptoms. Pursuing this observation in murine zymosan-induced peritonitis (D), T and B cells native to the naive peritoneal cavity were found to disappear within hours of stimulus injection. Once inflammation resolves, lymphocytes repopulate the peritoneum but comprise more (E-J) $CD4^+$/CD25 and γ/δ T cells as well as NK cells, than is present in the naive cavity (0 hours) as well as (K) MAC-1^+ B cells. (L) Experimentally enhancing the severity of the inflammatory response within the peritoneum by injecting 3 separate doses of zymosan (0.1, 1.0, and 10 mg) prolonged resolution and delayed lymphocyte repopulation (72 hours), suggesting that lymphocytes repopulate only after resolution occurs. n = 6 to 8 animals per group; $*P \leq .05$; $**P \leq .01$, as determined by analysis of variance (ANOVA), followed by Bonferroni *t* test, with data expressed as means plus or minus SEM.

peritoneum during or just after resolution occurs. Indeed, experimentally enhancing the severity of the inflammatory response within the peritoneum by injecting 3 separate doses of zymosan (0.1, 1.0, and 10 mg), therefore prolonging resolution, is associated with reduced lymphocyte repopulation (Figure 1L). Whether this is a delay or suppression is unclear at this stage. However, from the data presented here, we suggest that lymphocytes repopulate sites of inflammation once resolution occurs. Therefore, this questions what controls lymphocyte influx, which we suggest may be factors released by stromal and/or hematopoietic cells once restitution processes are under way. If so, lymphocyte repopulation is an active process and, until resolution occurs, lymphocyte repopulation is suppressed. Thus, from these experiments we show a shift in lymphocyte populations from the naive to a resolving state constituting more innate-type lymphocytes as well as a different phenotype of B1 cells.

Lymphocytes are dispensable for resolution but mediate responses to superinfection

To examine the role of lymphocytes in acute inflammation, zymosan was injected into the peritoneal cavity of lymphocyte-deficient $RAG1^{-/-}$ mice. Inflammation at onset was greatly exaggerated in these animals, twice that in wild-type (Figure 2A), with the principal cell type being PMNs (Figure 2B). This exaggerated response in RAG1 knockout mice was associated with decreased exudate IL-10 and elevated TNFα levels (Figure 2C). Surprisingly, inflammation in wild-type and $RAG1^{-/-}$ mice resolved uniformly from 24 hours onward (Figure 2A,B), suggesting that lymphocytes have no role in switching off acute inflammation and that regardless of how many PMNs traffic to sites of tissue injury, resolution pathways are sufficiently adept at dealing with their disposal. Pursuing the idea that lymphocytes may protect against secondary infection, in a second experiment, $RAG1^{-/-}$ and wild-type mice were injected with a sublethal dose of GBS 48 hours after zymosan injection. Thus, live bacteria were introduced into the inflamed cavity as inflammation resolved, and its effects determined 24 hours later (Figure 2D). Wild-type mice that got zymosan followed by bacteria displayed fewer signs of illness compared with those that received GBS alone, which exhibited 50% mortality by 24 hours, with the remaining animals dying by 48 hours. However, injecting GBS into $RAG1^{-/-}$ mice 48 hours after receiving zymosan showed an approximate doubling of inflammatory cell accumulation compared with wild-type mice treated in the same way (Figure 2D). This resulted in a lower bacterial load in the plasma of GBS-treated $RAG1^{-/-}$ (Figure 2E) but substantially accelerated mortality due to the concomitant hyperinflammatory response (Figure 2F). Results from these studies suggest that inflammation in the resolving phase exerts greater defense against bacterial infection and lethality than do naive tissues. In addition, in terms of controlling initial leukocyte trafficking in response to nonspecific stimuli, protection is conferred by resident lymphocytes, with resistance to secondary infection exerted by repopulating, resolution-phase lymphocytes. Therefore, lymphocytes modulate host responses to injury/infection but are not required to bring about resolution (ie, clear

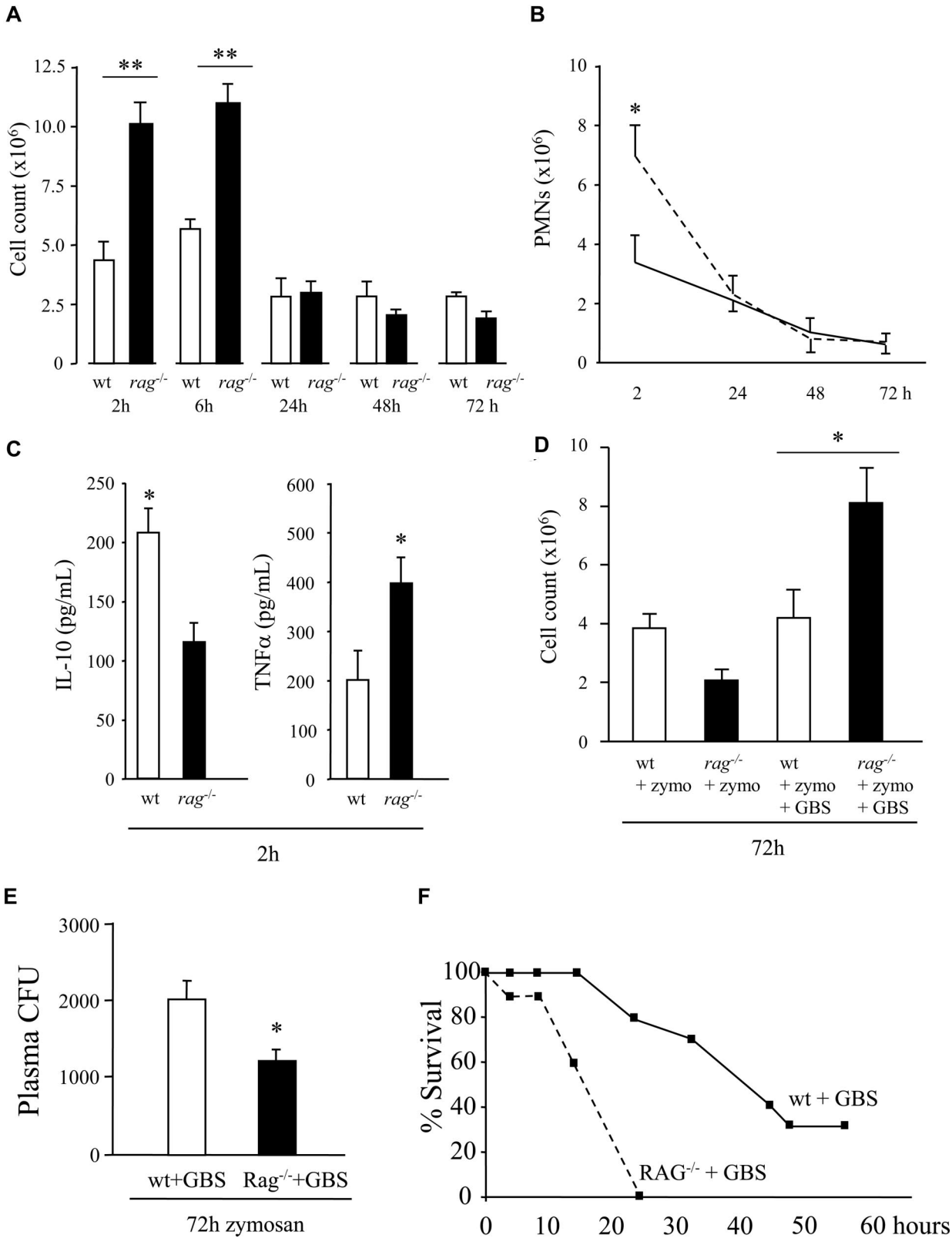

Figure 2. Lymphocytes control early onset of innate inflammation but are dispensable for its resolution. (A,B) Although inflammation doubled in RAG1$^{-/-}$ during the onset phase in response to zymosan, coincident with (C) an imbalance of IL-10 versus TNFα, inflammation normalized with that of wild-type mice from 24 hours onward, suggesting no role for lymphocytes in actively bringing about resolution (ie, clearing PMNs or macrophages). (D) However, RAG1$^{-/-}$ and wild-type mice were injected with GBS during resolution (48 hours after zymosan injection), resulting in enhanced leukocyte accumulation in RAG1$^{-/-}$ but not wild-type mice 24 hours later. (E) This was associated with reduced bacterial colonization in plasma but (F) increased mortality in RAG1$^{-/-}$ mice as a result of the concomitant hyperinflammatory response. We therefore argue that lymphocytes are not required for bringing about resolution and propose that their reappearance hails the end of the inflammatory event and an attempt at restorative physiology. Their role in this setting is in protecting against secondary infection or injury with B cells, CD4^{+}/CD25^{+} cells, and γ/δ T cells as well as NK cells playing a likely role in this setting. n = 6 to 8 animals per group, with experiments repeated on 2 separate occasions to confirm original findings. *$P \leq .05$; **$P \leq .01$, as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as means plus or minus SEM.

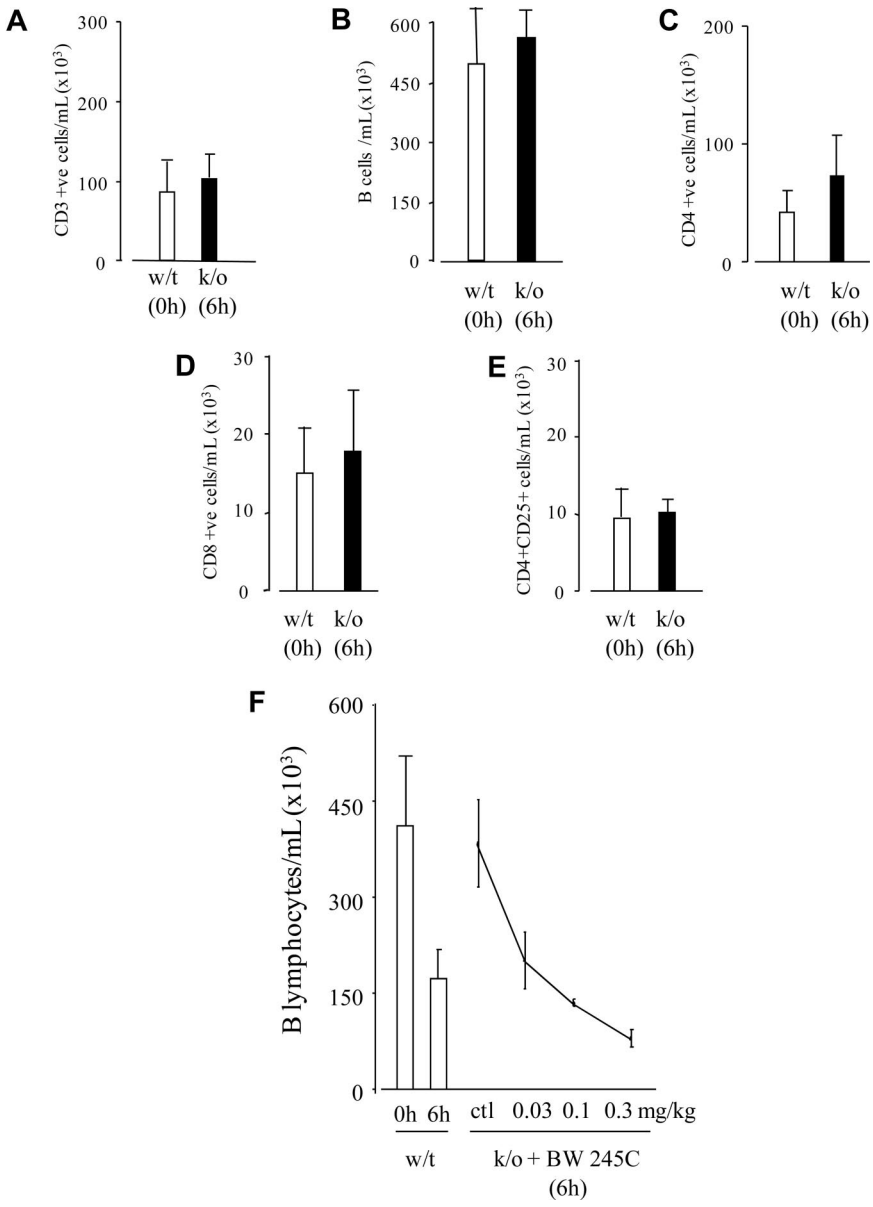

Figure 3. PGD_2 controls the clearance of peritoneal resident lymphocytes. In response to inflammatory stimuli, lymphocytes in the peritoneum disappear between 6 and 24 hours. (A-E) However, lymphocyte numbers in $hPGD_2S$ knockout mice at 6 hours (■) were found to be equivalent to that present in the naive cavity of wild-type mice (□), suggesting a role for either PGD_2 and/or 15d-PGJ_2 in the initial clearance of lymphocytes. (F) Adding back BW245C (DP1 receptor agonist) to $hPGD_2S$ knockout mice caused a reduction in B cells. Attempts made to identify the fate of CD3 cells generated inconclusive results, with data suggesting that they may die locally by programmed cell death (data not included). n = 8 animals per group. $^*P \leq .05$ as determined by Bonferroni *t* test, with data expressed as means plus or minus SEM.

PMNs and macrophages from inflamed sites). The different profile and proportion of lymphocyte repopulating during resolution is necessary to combat secondary infection.

Peritoneal lymphocytes disappear in response to PGD_2 STOPPED

Investigating how lymphocytes disappear, we recorded equivalent numbers of T and B lymphocytes in the peritoneal cavity of $hPGD_2S$ knockout mice at 6 hours as in the naive cavity (0 hours) of wild-type (Figure 3A-E). $hPGD_2S$ metabolizes cyclooxygenase-derived PGH_2 to PGD_2,[18] which activates 2 G-protein–coupled receptors, DP1 and DP2 (CRTH2).[19,20] PGD_2 is further dehydrated to the cyclopentenone PG, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2),[21,22] with levels of PGD_2 and 15d-PGJ_2 peaking within the first few hours of acute peritonitis coincident with lymphocyte disappearance.[11] Incidentally, the existence of 15d-PGJ_2 in mammalian systems has been hotly debated over the years, but in these experiments, we used liquid chromatography–mass spectrometry–mass spectrometry (LC-MS-MS) to definitively confirm its presence in resolving inflammation.[11] Adding BW245C (DP1 agonist[15,16]) to $hPGD_2S$ knockout mice reduced lymphocytes at 6 hours, with the clearance effects of DP1 on B cells (Figure 3F). 15(R)-15-methyl PGD_2 (DP2 agonist[17]) had no effect on lymphocyte numbers in knockout mice, indicating that DP1 receptor activation is responsible for B-cell disappearance early in acute inflammation. The fate of $CD3^+$ cells remains less clear. For instance, CD3 cells were examined for their adherence to the greater omental lymphoid organ, the so-called leukocyte disappearance reaction typical of peritoneal macrophages during acute peritonitis.[23] But, addition of 5% trypsin to a 4-hour inflamed cavity for 10 minutes recovered displaced macrophages but not lymphocytes (Figure S7). Another possibility was that CD3 cells underwent programmed cell death in response to 15d-PGJ_2, a highly reactive electrophile and, in our experience, a potent inducer of lymphocyte apoptosis. Although CD3 cells from $hPGD_2S^{-/-}$ mice remained low for annexin V/propidium iodide labeling up to 6 hours, on a percentage basis there was a trend toward an increase in annexin V labeling within the equivalent population in wild-type mice. However, this did not reach significance due to the diminished numbers of peritoneal CD3 cells in wild-type mice available

for analysis (data not included). Therefore, we can suggest that within a few hours of inducing an inflammatory response, peritoneal resident B cells disappear in a PGD_2/DP1-dependent manner, but that the fate of $CD3^+$ cells remains unclear.

Deficiency of repopulating lymphocytes in nonresolving chronic granulomatous disease

Finally, the relevance of these findings to human inflammatory diseases was determined by examining the role of lymphocytes in $gp91^{phox}$ knockout mice, an experimental model of human chronic granulomatous disease caused by defects in the phagocyte respiratory burst oxidase, which generates microbicidal superoxide.[24,25] Hence, patients with chronic granulomatous disease lack antimicrobial capacity and the ability to combat bacterial and fungal infections. Moreover, there is the associated occurrence of inflammatory granulomas in lung, liver, and skin, which in some instances may arise from sterile stimuli, suggesting that their formation may be due to incomplete degradation of inflammatory debris and/or impaired resolution of inflammation.[26,27] $gp91^{phox}$ knockout mice were injected intraperitoneally with sterile zymosan and found to have elevated leukocyte numbers compared with controls, with inflammation failing to resolve (Figure 4A). See also Figure S8 for comparison of cell types in both animals at resolution. Fewer lymphocytes were found at 48 to 96 hours in $gp91^{phox}$ knockout mice, the time frame of resolution and lymphocyte repopulation in wild-type mice (Figure 4B). Lymphocytes obtained from the resolution phase (72 hours) of normal strain-matched wild-type controls which were therefore composed of B1, NK, and γ/δ T cells as well as $CD4^+/CD25^+$ cells, were transferred back to $gp91^{phox}$ knockout mice (72 hours) and challenged with LPS. Inflammation was reduced in knockout mice that received resolution-phase lymphocytes compared with $gp91^{phox}$ mice alone (Figure 4C). These results suggest that during ongoing, nonresolving inflammation, the absence of lymphocytes may account for susceptibility to superinfection and the associated hyperinflammatory response.

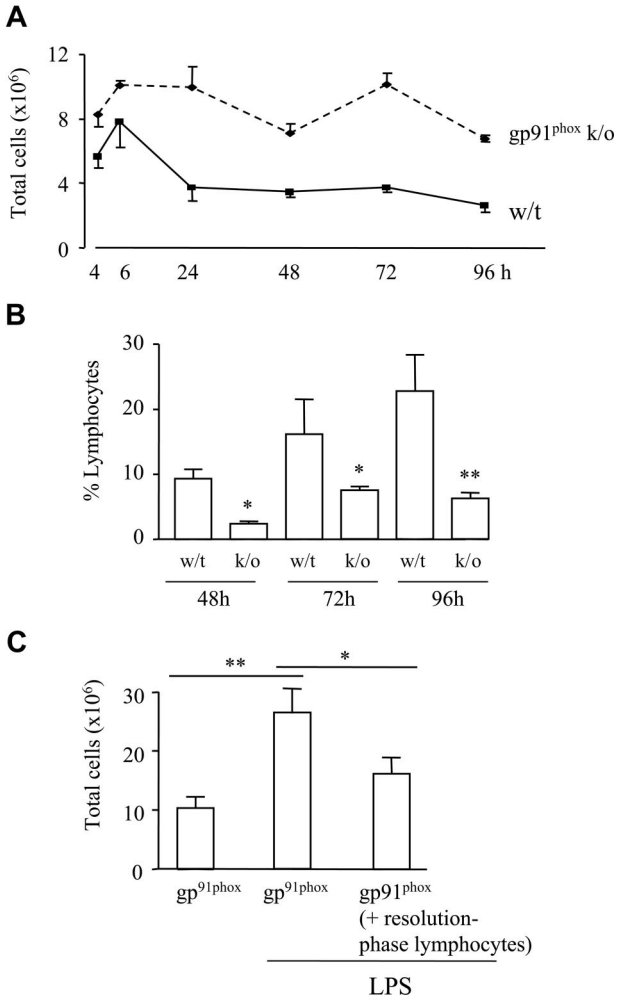

Figure 4. Absence of repopulating lymphocytes during nonresolving inflammation. (A) Zymosan was injected into the peritoneal cavity of $pg91^{phox}$ knockout mice, which, when compared with controls, showed a more aggressive inflammatory response that failed to resolve. (B) FACS analysis of cell types present during resolution revealed a progressive repopulation of lymphocytes during resolution that was lower in $pg91^{phox}$ knockout mice. (C) Lymphocytes obtained from the resolution phase (72 hours) of normal strain-matched wild-type controls and comprising B1 cells, NK cells, and γ/δ T cells, as well as $CD4^+/CD25^+$ cells, were transferred back into the peritoneal cavity of $gp91^{phox}$ knockout mice (72 hours) and subsequently challenged, intraperitoneally, with LPS. Inflammation was reduced in $gp91^{phox}$ knockout mice that received resolution-phase lymphocytes compared with $gp91^{phox}$ mice alone. $^*P \leq .05$; $^{**}P \leq .01$ as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as means plus or minus SEM.

Discussion

Here, evidence is presented that lymphocytes play a pivotal role in controlling the onset of innate immune-mediated inflammation by regulating cytokine synthesis as well as host susceptibility to secondary infection. Analysis of lymphocyte subsets in the naive peritoneal cavity of mice revealed that B cells constitute about 70% to 80% of the total lymphocyte population, of which the majority have a B1 phenotype, with the remainder being B2 cells. $CD3^+$ cells as well as NK and γ/δ cells make up the remaining 20% of lymphocytes. This profile differs to that found at resolution, which comprises more innate-type lymphocytes and B1 cells expressing MAC-1. Although we did not discern which lymphocyte or combination of lymphocytes bestowed protection at onset or at resolution, in a separate study, peritoneal CD3 T cells and in particular $B220^+$ B cells were found to elaborate high levels of IL-10 in a DP1-dependent manner,[11] most likely explaining the reduction in IL-10 in lymphocyte-deficient $RAG1^{-/-}$ mice and subsequent increase in PMN influx in these animals. Thus, given the relative proportion of T cells versus B1 cells in the inflamed cavity and the ability of B cells to synthesis comparatively high levels of IL-10, we suspect that B lymphocytes may one of the predominant cell types modulating acute inflammatory responses to nonspecific stimuli.

As PMNs begin to accumulate in response to zymosan, B cells disappeared in a PGD_2-dependent manner, as there was equivalent numbers of B cells in the inflamed cavity of $hPGD_2S^{-/-}$ at 6 hours as there was in the naive cavity of wild-type mice. This accumulation of B cells in $hPGD_2S$ knockout mice was reversed by BW245C, a DP1 receptor agonist, with DP2(CRTH2) playing no role in this setting. While the mechanism of PGD_2-dependent B-cell clearance is unknown, a Toll-like receptor (TLR)–mediated transient down-regulation of integrins and CD9 on B1 cells was shown to be required for detachment of these cells from the local peritoneal matrix and their subsequent efflux from the inflamed cavity.[28] Whether BW245C alters CD9 expression needs further investigation, but given that B cells disappear concomitantly with PGD_2 synthesis, it is possible that DP1 activation may play a role in regulating this pathway of B1 cell detachment and efflux. The fate of CD3 cells, on the other hand, is less clear. While their attachment to the peritoneal lining can certainly be excluded, there is the

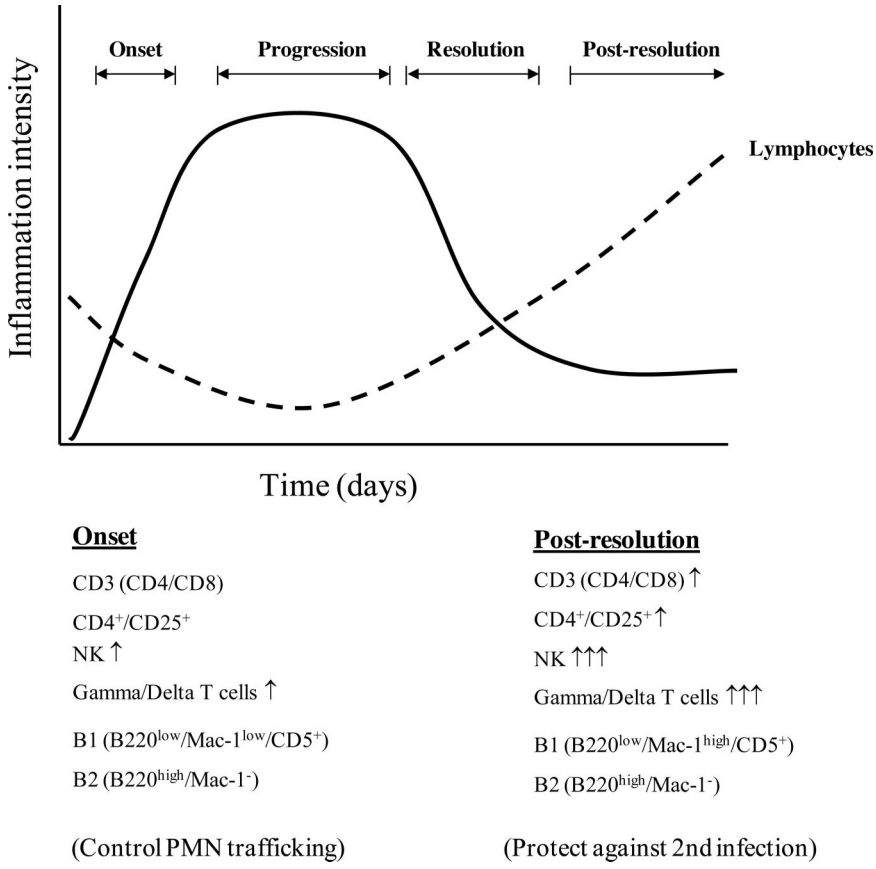

Figure 5. A summary of the scheme of events that occurs in acute inflammation with reference to lymphocyte trafficking. As inflammation ensues resident lymphocytes begin to disappear, with B1 cells clearing via draining lymphatics and the fate of CD3 cells remaining unclear at this stage. Once inflammation begins to resolve, lymphocytes repopulate the site of injury in a profile different to that in the naive state.

possibility that these cells may die locally by programmed cell death in response to 15d-PGJ_2, which is synthesized concomitant with their disappearance,[11] and in our experience, a potent inducer of lymphocyte apoptosis. However, future detailed work is required to definitively identify whether they die locally or clear via draining lymphatics. Thus, PGD_2 exerts a dual role on resident B cells at least—regulating their inflammatory cytokine release as well as their efflux from the inflamed peritoneal cavity.

The mechanism by which lymphocytes exert their protective effects in these experiments is unclear at this stage. Certainly, there is a cytokine imbalance favoring proinflammatory TNFα but reduced IL-10 in the inflamed peritoneum of RAG mice, thereby potentially explaining the enhanced influx of PMNs in these mice compared with wild-type. Indeed, we have shown that both T and B lymphocytes are capable of elaborating inflammatory cytokines, which, on balance, in the inflamed peritoneum at least, serve to limit PMN influx.[11] However, in addition to cytokines/chemokines, cell adhesion molecules are also central to facilitating PMN adhesion and accumulation at sites of inflammation and, contrary to data presented here, there is evidence showing that lymphocytes trigger cell adhesion molecule expression. For instance, intracellular adhesion molecule 1 expression in *Plasmodium*-infected mice is reduced in the brain but not the lung of $RAG1^{-/-}$ mice, while P-selectin expression is attenuated in both organs in these animals.[29] Equally, T cells were shown to enhance the expression of TNFα-triggered endothelial cell adhesion molecule expression, with these effects varying between vascular beds.[30] On this basis, it is difficult to reconcile the role circulating lymphocytes play in up-regulating cell adhesion molecule expression on the leukocyte/endothelial cell interface to that of the resident peritoneal T/B cells other than to highlight the different profile of lymphocytes present in the peritoneum (B1, B2, and small numbers of CD4/CD25 cells) that exert a predominantly protective effect in both the naive and postresolution state.

The trigger for lymphocyte repopulation is unclear, but critical determinants of resolution such as PMN apoptosis or signals released by macrophages during phagocytosis of apoptotic leukocytes may play a central role. However, we have shown previously that inducible cyclo-oxygenase is expressed during and is essential for the resolution of acute inflammation,[6,31,32] while others have reported that lipoxygenase-lipoxygenase interaction products of arachidonic as well as eicosapentaenoic and docosahexanoic acids dampen the severity of inflammatory onset and trigger resolution.[33-35] Taking a closer look at whether cyclo-oxygenase or lipoxygenase play a role in lymphocyte repopulation, we found that not only is COX-2 expressed during the resolution phase of zymosan-induced peritonitis, but that its inhibition with either NS-398 or the nonselective COX inhibitor indomethacin impairs lymphocyte repopulation, in particular $CD3^+$ cells. However, inhibition of lipoxygenase isoforms with baicalein was without effect. These data implicate a COX-2 metabolite in the recruitment of postresolution lymphocytes and therefore protection against host susceptibility to superinfection (J. Newsom, M. Stables, P.C.-N., G.B., and D.W.G., manuscript in preparation).

Comparing repopulating lymphocytes with those in the naive cavity revealed more NK cells, γ/δ T cells, and $CD4^+/CD25^+$ cells, in addition to B1 cells with higher Mac-1 labeling than that found at onset. The functional relevance of increased MAC-1 expression on resolving B1 cells is not apparent at this stage, but may reflect a state of differentiation/activation or a specific requirement for migration back to the resolved peritoneum. However, repopulating lymphocytes have no role in actively bringing about resolution, but

protect against superinfection. In these experiments, GBS was given into the resolving peritoneal cavities of wild-type as well as RAG1$^{-/-}$ mice. Interestingly, the degree of inflammation in wild-type mice that received zymosan followed by GBS was not significantly greater than the level of inflammation in resolving wild-type mice not given GBS. In contrast, inflammation in RAG1$^{-/-}$ mice that received zymosan followed by GBS was almost twice that of inoculated wild-type mice. This suggests that resolution-phase lymphocytes confer protection against secondary infection. This was certainly confirmed by injecting live bacteria to RAG$^{-/-}$ mice undergoing resolution, which subsequently died substantially faster than wild-type mice treated in the same manner (Figure 2F). It is unknown why the proportion and profile of repopulating lymphocytes is different to that at onset. Perhaps as resolving tissues are physiologically altered as a consequence of the inflammatory event they underwent, host defense mechanisms need to be fundamentally different to guard against superinfection by recruiting more protective lymphocytes. On this theme, it is not really understood when acutely inflamed tissues revert back to their original state. Certainly, a population of macrophages (about 1×10^6) were found to linger for at least 3 weeks after a zymosan peritonitis apparently resolved, supporting the idea that although the original response was acute and transitory in terms of PMN influx and efflux, its effects may be longer-lasting than originally believed, thereby requiring a different profile and greater proportion of lymphocytes to modulate future inflammatory events. This may explain why inflammation in the resolving phase conferred greater protection against GBS lethality than uninflamed or naive mice (Figure 2D,F).

Taking these findings to a more clinically relevant setting, it became clear that unlike wild-type mice, there was a deficit of repopulating lymphocytes in nonresolving gp91^{phox} knockout mice bearing zymosan-induced peritonitis. Replenishing gp91^{phox} knockout mice with resolution-phase lymphocytes taken from strain-matched controls and then challenging animals with LPS conferred protection compared with sham-operated gp91^{phox} knockout mice. Data from this study confirm that not only does lymphocyte repopulation fail to occur in nonresolving inflammation, but that resolving-phase lymphocytes protect against exaggerated inflammatory responses to superinfection (Figures 2D,4C). On this note, defects in innate lymphocyte functioning have been suggested to lead to secondary infections associated with burn injury,[36] while increased lymphocyte apoptosis contributes to the pathogenesis of sepsis,[4,5] underlining the crucial role lymphocytes play in host defense against nonspecific injury. Moreover, as NK cells were originally described for their ability to lyse tumor cells,[37] and γ/δ T cells have well-known tumor surveillance properties,[38,39] their absence from sites of nonresolving inflammation may be one of the predisposing factors to the development of inflammation-related cancer.[40] Thus, one of the hazards of ongoing acute inflammation and consequently failed lymphocyte repopulation may be increased susceptibility to superinfection and even cancer as a result of lymphocyte apoptosis, lymphocyte immunoparalysis, or, as presented here, a failure of protective lymphocytes to repopulate after resolution.

In summary, we report a biphasic role for lymphocytes during innate immune-mediated inflammation, summarized in Figure 5. The first phase controls PMN trafficking with lymphocytes then vacating the peritoneal cavity in response to PGD_2 activating its DP1 receptor. The second phase is characterized by lymphocyte repopulation occurring after inflammation begins to resolve in a different proportion and profile to that of the naive state. Importantly, repopulating lymphocytes have no role in bringing about resolution, but protect against secondary infection.

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Authorship

Contribution: D.W.G. designed research and wrote the paper along with R.R., who carried out the research. T.L., G.B., J.B., and P.C.N. carried out experiments and provided essential experimental material. M.H., D.F., and M.M.Y. supplied essential experimental tools. D.W.G. and P.C.-N. analyzed data.

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Nonresolving Inflammation in gp91phox$^{-/-}$ Mice, a Model of Human Chronic Granulomatous Disease, Has Lower Adenosine and Cyclic Adenosine 5′-Monophoshate

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Abstract

In chronic granulomatous disease (CGD) there is failure to generate reactive oxygen metabolites resulting in recurrent infections and persistent inflammatory events. As responses to sterile stimuli in murine models of CGD also result in non-resolving inflammation, we investigated whether defects in endogenous counter-regulatory mechanisms and/or pro-resolution pathways contribute to the aetiology of CGD. To this end we carried out a series of experiments finding, in the first instance that adenosine and cAMP, which dampen innate immune-mediated responses, show a biphasic profile in resolving peritonitis; peaking at onset, waning as inflammation progresses and rising again at resolution. We also found elevations in adenosine and cAMP in resolving human peritonitis. In gp91phox$^{-/-}$ mice, an experimental model of CGD, levels of adenosine and cAMP were significantly lower at onset and again at resolution. Corroborating the finding of others, we show that adenosine, signalling through its A_{2A} receptor and therefore elevating cAMP is not only anti-inflammatory but, importantly, it does not impair pro-resolution pathways, properties typical of nonsteroidal anti-inflammatory drugs. Conversely, antagonising the A_{2A} receptor worsens acute inflammation and prolongs resolution. Taking this further, activating the A_{2A} receptor in gp91phox$^{-/-}$ mice was dramatically anti-inflammatory regardless of the phase of the inflammatory response A_{2A} agonists were administered i.e. onset or resolution demonstrating wide and robust pharmacological flexibility that is unlikely to subvert pro-resolution pathways. Therefore, we describe the biphasic profile of adenosine and cAMP throughout the time course of acute inflammation that is dysregulated in CGD.

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Keywords

Lipid mediators; Cytokines; Human peritonitis

INTRODUCTION

In response to injury/infection there is the sequential release of pro-inflammatory mediators including complement(1), histamine(2,3), bradykinin(4) and 5-hydroxytryptamine(5) along with cytokines and chemokines(6) that cause cell and edema accumulation. Concomitantly, there are braking signals that temper the severity of this early onset phase including adrenaline (7), adenosine(8-10), steroid hormones(11), cAMP(12) and counter-regulatory cytokines including IL-10(13). In attempts to enhance our understanding of acute inflammation, we are beginning to realise that, in addition to endogenous checkpoints that temper PMN trafficking there are also signals that control the transition of PMNs to phagocytosing macrophages (prostaglandin [PG]E_2(14), IL6/IL6ra complex(15)), scavenge cytokine/chemokine from the inflammatory environment (D6 scavenger receptor(16), lipoxins(17)) as well as clear macrophages to the draining lymph nodes (PGD_2(18) and resolvins(19)) leading to inflammatory resolution. Advancing this paradigm even more, our group recently identified the post-resolution influx of innate-type lymphocytes to sites of inflammation with a role in controlling host responses to secondary inflammation and superinfection(20). Indeed, we propose, for the first time, that one of the cardinal signs of resolution is the recruitment of innate-type lymphocytes to sites of tissue injury concomitant with inflammation switching off. It is argued that while inflammation has apparently resolved, classically defined by the clearance of inflammatory leukocytes, there are events occurring locally involving innate-type lymphocytes that have an impact on organ and host health long after inflammation has abated. Thus, innate immune responses are designed to combat infection and repair injured tissues in a timely manner. However, there are instances when the endogenous factors that control immune severity become dysregulated, resulting in progression to chronic inflammation and tissue injury. To combat this, anti-inflammatory agents were designed based on their inhibition of signals that propagate inflammation(21) with little consideration for any adverse impact on pro-resolution pathways, as has been demonstrated with non-steroidal anti-inflammatory drugs (NSAIDs)(22). Consequently, when treating inflammation we must also aim to trigger its resolution or, at least, not impair endogenous pathways that lead to tissue homeostasis(23), with one of the hallmarks for this, in the peritoneum at least, being innate-type lymphocyte repopulation(20).

There are many clinical examples where chronic inflammation could be hypothesised to be potentially derived from dysregulated resolution pathways. Thus, a human disease that fails to resolve and for which there is an experimental animal model amenable to scientific interrogation, would aid enormously in elucidating the aetiology of chronic inflammation. To this end, we turned to chronic granulomatous disease (CGD), which is an inherited immunodeficiency syndrome caused by a defect in the oxygen metabolic-burst machinery resulting in the inability to neutralise infection leading to persistent and recurrent inflammatory responses and granulomatous tissue formation(24). Activity of NADPH oxidase system (gp91phox, p22phox, p47phox and p67phox) is either absent or dysregulated in these patients with the most common being X-linked CGD (~65%) with defects in the gene encoding gp91phox. Fortunately, for the purpose of understanding the aetiology of CGD, gp91phox deficient mice display all the hallmarks of the human condition in response to infection(25). Interestingly, there are reports showing that inflammation in CGD mice is also prolonged and dysregulated in response to sterile stimuli(26,27), suggesting potential irregularities in endogenous anti-inflammatory and/or pro-resolution pathways. We carried out a series of studies in gp91phox$^{-/-}$ mice and found that of the factors known to control resolution, few

appeared to be consistently dysregulated with the exception of adenosine and cAMP, levels of which are significantly lower in experimental CGD compared to wild types.

Under physiological conditions, adenosine is continuously formed intracelluarly and extracellularly. The intracellular production is mediated either by an intracellular 5′-nucleotidase, which dephosphorylates AMP or by hydrolysis of *S*-adenosyl-homocysteine (28). Adenosine generated within cells is transported into the extracellular space *via* bi-directional transporters through facilitated diffusion that efficiently equilibrates intracellular and extracellular levels of adenosine. Following trauma, there is a decrease of intracellular ATP, accompanied by an accumulation of 5′-AMP and subsequently adenosine by the above pathways, which may be sequentially metabolized to inosine, hypoxanthine and xanthine. Expressed on cells of the hematopoietic system, adenosine receptors (A_1, A_{2A}, A_{2B}, A_3) belong to the family of G-protein-coupled heptahelical transmembrane receptors, which either stimulate (Gs) or inhibit (Gi) adenylyl cyclase, the enzyme that catalyzes the formation of cAMP(28). Adenosine A_1 and A_3 receptors are high and low affinity receptors for adenosine, respectively, with both being inhibitors of adenylyl cyclase. High-affinity A_{2A} and low-affinity A_{2B} receptors, on the other hand, activate adenylyl cyclase, thereby increasing intracellular levels of cAMP, resulting in potent immune-suppression and regulation of inflammatory leukocyte trafficking. Besides controlling adenylyl cyclase, adenosine receptors are also coupled by distinct G-proteins to several other effector systems, including calcium and potassium channels, phospholipase C, D, A2, cGMP, phosphodiesterases, and mitogen-activated protein kinases that modulate different cell functions. Thus, adenosine, released after tissue injury or low oxygen tension associated with inflammation, has been regarded by some to act as a first line sensor of immune damage where it prevents further damage by inhibiting activated immune cells with its immune-suppression mediated by A_{2A} receptor elevation of cAMP(10,29).

In this study we report the biphasic synthesis of both adenosine and cAMP, first at the traditional early onset phase of acute inflammation and again during resolution, with synthesis of these immunosuppressive agents being significantly lower in CGD (gp91phox$^{-/-}$) mice associated with a severe and prolonged innate immune response to a sterile stimuli. We also show that hyper-inflammation in gp91phox$^{-/-}$ mice can be rescued by A_{2A} receptor activation as defined by reduction in inflammatory leukocytes. Importantly, A_{2A} receptor activation in gp91phox$^{-/-}$ mice did not bring about resolution as this drug strategy was not associated with innate-type lymphocyte repopulation that is typical of events that occur during normal resolution in wild type leading to tissue homeostasis.

MATERIALS AND METHODS

Animal maintenance, induction of inflammation and human peritonitis sampling

gp91phox$^{-/-}$ mice (Jackson Laboratories, Maine, USA),along with wild type mice, were bred under standard conditions and maintained in a 12h/12h light/dark cycle at 22 ± 1°C and given food and tap water *ad libitum* in accordance with United Kingdom Home Office regulations. The murine 7-day air pouch was elicited by the injection of 3ml of sterile air followed 7 days later with the intra-pouch injection of 0.5ml 1% carrageenin. Peritonitis was induced by the intraperitoneal injection of 1mg type A zymosan (Sigma) and cells enumerated by haemocytometer at time points stated in results section by sterile PBS washout. Ethical approval (P/03/136A) for collection of human peritonitis samples was obtained from St. Bartholomew's & the Royal London Hospitals from end stage renal failure patients undergoing peritoneal dialysis.

Macrophage isolation, culture and stimulation

Peripheral venous blood samples were collected from subjects into heparinised syringes (5U/ml). Mononuclear cells were isolated by differential centrifugation (2000rpm, 30 mins, 20°C) over Lymphoprep® (Axis-Shield, Oslo, Norway) and washed twice with sterile phosphate-buffered saline (PBS) (GIBCO, Paisley, UK) at 1200rpm (5 mins, 20°C). Cells were re-suspended in 10mls RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 100U/ml of penicillin (GIBCO) and 100μg/ml streptomycin (GIBCO) and 20mM Hepes buffer (Sigma) (RPMI), and plated at a density of approximately 5×10^6 cells/ml in $8cm^2$ Nunclon™ Surface tissue culture dishes (Nunc, Roskilde, Denmark). After an initial culture period of 2 hours at 37°C, 5% CO_2, the non-adherent cells were discarded and 10mls of fresh RPMI supplemented with 10% foetal bovine serum (Sigma) (10% FBS/RPMI) added to each tissue culture dish. Cells were then cultured for 5 days at 37°C, 5% CO_2, with the addition of a further 10mls fresh 10% FBS/RPMI after 24 hours. Adherent cells were scraped on day 5 and re-plated in 96-well culture plates at equal densities (10^5/well) in X-Vivo-15 medium (Cambrex, Walkersville, MD, USA). These primary monocyte-derived macrophages were incubated overnight at 37°C, 5% CO_2 to adhere, and then stimulated for 24 hours with 200ng/ml lipopolysaccharide (LPS) (Alexis, San Diego, CA, USA). These studies were approved by the Joint UCL/UCLH Committees on the Ethics of Human Research (02/0324). Written informed consent was obtained from all volunteers. No patient was studied more than once in each of the different sets of experiments.

Pharmacological rescue experiments

gp91phox$^{-/-/-}$ and wild type mice were pre-treated (30mins) with the A_{2A} receptor agonist CGS 21680 (2mg/kg) with/without ZM241385 (2mg/kg, A_{2A} receptor antagonist) followed by zymosan intraperitoneally. Rolipram was dosed at 30mg/kg. The peritoneal cavity was lavaged at 4 hours and cell number counted using a haemocytometer. For experiments in the resolution phase, inflammation was firstly induced with zymosan followed by CGS 21680 or vehicle (DMSO) at 24 and 36 hours. The peritoneal cavity was lavaged at 48 hours following zymosan. Caffeine as well as its stable analogue, 8-(3-chlorostyryl)-caffeine (CSC) were dosed at 3mg/kg.

Eicosanoid analysis

Samples stored at -20°C were thawed at room temperature and acidified to pH 3. Samples were extracted using C18 columns (Waters). For PGD_2, samples were then treated with methoxylamine hydrochloride (MOX HCL) and the resulting stable PGD_2-MOX measured by EIA (Cayman Chemicals, USA). PGE2 was measured by EIA (Cayman Chemicals, USA) while lipoxin A4 was quantified by ELISA (Neogen, USA).

Purine and cAMP measurements

Proteins in exudates were removed by ultrafiltration (30,000 Dalton cut-off). Purine concentrations in samples were measured by high pressure liquid chromatography as previously described(30).

FACS analysis and cytokine/chemokine analysis

Cytokines were measured by ELISA according to manufacturer's instructions (eBiosciences). FACS was carried out on Becton Dickinson Facscalibur with data analysed by Cellquest. Leukocytes were incubated with antibodies for 30mins to either CD3/CD19 (Serotec, UK), B cells (Ly220, Serotec), GR1 (BD Pharmingen, UK) or F4/80 (Caltag laboratories, CA, USA) using respective isotype antibodies as controls (Serotec, UK) and compensated as appropriate for dual labelling. For apoptosis, cells were incubated with annexin V/propidium iodide

(Becton Dickinson) and analysed on Becton Dickinson Facscalibur with data analysed by Cellquest.

RESULTS

Inflammation is more severe and fails to resolve in gp91phox$^{-/-}$ mice

In the first set of experiments we characterised the profile of inflammation in wild types versus gp91phox$^{-/-}$ mice. Zymosan injection into the mouse peritoneal cavity resulted in an exaggerated influx of PMNs (Figure 1A, Ly6G positive cells) in knockouts during the early onset phase with numbers declining up to 96h. F4/80 positive macrophage numbers were similarly increased at onset in knockouts with levels remaining elevated throughout the entire response, Figure 1B, underlining the non-resolving nature of inflammation that is characteristic of CGD. As there was little difference in leukocyte apoptotic rates as determined by annexin V/propidium iodide labelling between both animals, Figure 1C, failure of resolution in CGD mice most likely resulted from continual influx and/or failed clearance of inflammatory leukocytes.

Reduced synthesis of purines and cAMP in gp91phox$^{-/-}$ mice

The classic view as to why inflammation is more severe in CGD is explained by defects in phagocytic oxidase resulting in impaired bacterial killing and consequently delayed removal of the injurious agent. That notwithstanding, injection of sterile inflammatory stimuli also results in an exaggerated inflammatory event that fails to resolve(26,27). Certainly, while this may arise from a defect in phagocytosis(31), we questioned whether it may also point to a possible defect in endogenous braking systems that counter-regulate acute inflammation(23, 32,33). To this end we screened for alterations in levels of anti-inflammatory and pro-resolution mediators finding no consistent trend in arachidonic acid metabolism between CGD and wild type mice bearing a zymosan-triggered peritonitis. For instance, PGD_2(18,22,34), the lipoxins (35) and PGE_2(14) have all been shown to trigger inflammatory resolution, yet, with the exception of PGE_2 there was little evidence for defects in arachidonic acid metabolism being involved in aetiology of CGD, Figures 2A-C. However, we noted a clear and robust reduction in levels of cAMP in CGD mice compared to wild type animals, Figure 2D. Specifically, intracellular cAMP was elevated during the early onset phase of zymosan-induced peritonitis in wild type controls (4-6h), waning as inflammation progressed and became elevated again post-resolution, Figure 2D. In gp91phox$^{-/-}$ mice bearing a zymosan-triggered peritonitis, cAMP was significantly lower than wild types at onset and failed to show the post-resolution elevation seen in wild types, Figure 2D. The functional relevance of raised cAMP post resolution is being answered in another body of work (paper enclosed), which shows that cAMP controls the phenotype of resolution-phase macrophages imparting upon them an immunosuppressive state. We next examined why cAMP levels are lower in gp91phox$^{-/-}$ mice. Certainly, PGs are well known elevators of cAMP (*via* EP2, EP4, DP1)(36) and are also present and functional during the early as well as later stages of acute inflammation(22). Nonetheless, there was no substantial difference in cyclooxygenase activity between wild types and gp91phox$^{-/-}$ mice. In fact, there was an elevation of PGE_2 (Figure 2A) in gp91phox knockouts indicating possible signalling of this PG through its EP1 (increased Ca2+) and/or EP3 (IP_3/DAG) receptors in CGD mice. However, there was a biphasic profile of adenosine synthesis mirroring that of cAMP - raised at onset and then again at resolution, Figure 2E. Adenosine showed lower levels in gp91phox$^{-/-}$ mice than wild types and is a well described immuno-modulator of acute inflammation serving to dampen PMN function and prevent chemical-induced collateral liver injury(37). With its molecular actions exerted through four receptors A_1, A_2 (A_{2A} and A_{2B}) as well as A_3, signalling through A_{2A} has received most attention as a trigger for immuno-suppressive cAMP. Thus, from these studies we report the biphasic synthesis of adenosine and cAMP in resolving models of acute inflammation, with their levels

diminished in inflammation associated with CGD. It could be argued that it is the absence of these endogenous anti-inflammatories in CGD that is responsible for its hyper-inflammatory, non-resolving nature. Alternatively, persistence of the inflammatory stimulus would certainly prevent resolution and consequently deactivate or override endogenous pro-resolution pathways. The latter may indeed be the case as levels of cAMP released from monocyte-derived macrophages obtained from CGD patients released similar quantities of cAMP per equivalent cell numbers to those from healthy volunteers when stimulated with LPS, Figure 2F.

Adenosine and cAMP profiles in human resolving peritonitis

As an alternative to dialysis, patients on end-stage renal failure may undergo chronic ambulatory peritoneal dialysis (CAPD) where a catheter is inserted to fill/drain the peritoneal cavity with a high glucose solution with the peritoneal lining acting as a dialysing membrane. Occasionally, patients experience bacterial infection (commonly *S. aureus* or *S. epidermidis*) resulting in acute resolving peritonitis from which both effluent and cells can be analysed for markers of inflammation and resolution(38). We measured levels of cAMP (Figure 3A) as well as adenosine, (Figure 3B) in these samples and found that when overlaid on the inflammatory leukocyte profile (Figure 3C), which peaked 24h after infection, cAMP as well as adenosine was elevated as inflammation resolved. While these results corroborates that found in mice, we have no data on the very early onset (~ 6h) phase in humans to assess the very early release of adenosine and cAMP in human inflammation. Thus, as in rodents, levels of cAMP and adenosine were elevated as inflammation resolved.

Adenosine, *via* A_{2A} is anti-inflammatory and not resolution-toxic

As with the plethora of effects NSAIDs exert on acute inflammation, it is well established that by signalling through its A_{2A} receptor, adenosine exerts protective effects during acute inflammation at multiple levels. However, while NSAIDs dampen the early onset phase of acute inflammation, they also obstruct pro-resolution processes(22). Therefore, we next determined whether A_{2A} signalling interferes with inflammatory resolution. In the first instance, rats bearing a carrageenin-induced pleurisy were administered rolipram, a PDE4 inhibitor and therefore elevator of cAMP as well as CGS 21680, a specific A_{2A} receptor agonist 30mins prior to carrageenin injection. Both drugs dampened leukocyte trafficking to the inflamed cavity at onset i.e. 4h (Figure 4A), being associated with an expected rise in cAMP, Figure 4B. In a mouse zymosan-induced peritonitis at 4h, CGS 21680 also dampened inflammation in an A_{2A} receptor dependent manner using ZM 241385 (A_{2A} receptor antagonist) (Figure 4C) associated with a significant increase in anti-inflammatory IL-10, Figure 4D. Other drugs, including theophylline as well as beverages are known to alter cAMP signalling and therefore may unwittingly affect inflammation. A single cup of coffee, for instance, contains about 100mg of caffeine implying that an average person drinking one cup of coffee per day will ingest caffeine at 1.5mg/kg. To investigate whether caffeine, a methylxanthine with antagonistic effects on the A_{2A} receptor(39) affects acute inflammation, we administered caffeine as well as its stable analogue, 8-(3-chlorostyryl)-caffeine (CSC) at 3mg/kg 30mins before i.p. zymosan injection and found that both worsened inflammation possibly by decreasing protective IL-10 levels, Figures 4E and F. Using a continual pharmacological dosing regimen, mice were administered not only 30mins before zymosan injection but were also given caffeine and CSC again at 12h and 18h after zymosan and their effects assessed at 24h. The idea being that as inflammation resolves by 24h, any resolution-toxic effects of these drugs would be detected, an important consideration with anti-inflammatories that must be highlighted. CGS 21680 maintained a dampening of acute inflammation without interfering with resolution, Figure 4G. On the other hand, caffeine at doses representative of reasonable caffeine daily intake as well as its analogue, CSC, maintained their pro-inflammatory effects, Figures 4G. Taking the doses of caffeine up to 10 and 30mg/kg, equivalent to 6-20 cups of coffee, resulted in a loss of pro-inflammatory effects.

Therefore, from these experiments, A_{2A} agonists exert anti-inflammatory effects not only during the early onset phase of acute inflammation, the phase traditionally tested experimentally for novel anti-inflammatories, but, importantly do not interfere with pro-resolution pathways. An interesting observation was the corollorary to these experiments, which revealed that caffeine has antagonistic effects on endogenous protective pathways and is not only pro-inflammatory but potentially anti-resolution.

Adenosine *via* A_{2A} is anti-inflammatory but not pro-resolving in gp91phox$^{-/-}$ mice

Being anti-inflammatory is not the same as possessing pro-resolution properties(23). Therefore, in these final experiments, we determined whether drugs that signal through A_{2A} and raise cAMP rescued the hyper-inflammatory phenotype typical of gp91phox$^{-/-}$ mice and importantly, whether they bring about resolution of inflammation in these animals. Thus, CGS 21680 was dosed orally 30mins before zymosan to gp91phox$^{-/-}$ and wild types with inflammation assessed 4h later. Data revealed that leukocyte influx was greater in knockouts than wild types and that A_{2A} receptor activation in knockouts reversed inflammation back to levels seen in drug-treated wild types (Figure 5A), with the principle effect being on PMN numbers, Figure 5B. We then investigated whether CGS 21680, given therapeutically during the equivalent of resolution in wild types, could alter the progression of inflammation in gp91phox$^{-/-}$ mice. In wild types, CGS 21680, given at 24h and again at 36h after established inflammation, had surprisingly no effect on leukocyte numbers at 48h i.e. CGS 21680 was neither anti-inflammatory nor resolution-toxic in normal animals, Figure 5C. Interestingly, an identical dosing regime in knockouts revealed that CGS 21680 was not only anti-inflammatory in these animals but that it lowered inflammation below that of controls (Figure 5C) concomitant with an elevation of cAMP, Figure 5D. To determine whether this was an anti-inflammatory or a pro-resolution effect, we next quantified the number of lymphocytes in CGS 21680-treated gp91phox$^{-/-}$ mice. The rationale for using these criteria for resolution stemmed from our recent findings, which revealed that as inflammation resolves, lymphocyte repopulate the peritoneal cavity(20). Lymphocyte repopulation is not required to bring about resolution, but is critical in restoring tissue homeostasis and conferring protection against superinfection. In CGS 21680-treated gp91phox$^{-/-}$ mice, while there was a classic anti-inflammatory effect, reducing predominantly PMN numbers, we argue that this is not a resolution effect as repopulation lymphocytes were not seen, data not included.

DISCUSSION

Here we report an immediate increase in adenosine and cAMP at the early onset phase of acute inflammation that wanes as the response progresses only to increase again as inflammation resolves with levels being significantly lower in experimental CGD (gp91phox$^{-/-}$ mice). Whether this is a direct result of an interaction between NADPH oxidase systems and adenosine synthesis is unlikely as levels of cAMP in isolated cells from CDG patients produce similar levels, on a cell-for-cell basis, to normal healthy volunteers. Therefore, does the persistent nature of CGD arise from an inherent defect in counter-regulatory/pro-resolution pathways or a much simpler explanation of CGD patients being incapable of clearing inflammatory stimuli resulting in a persistent, almost frustrated innate immune response that consequently nullifies endogenous protective pathways? As emphasised previously one of the most critical determinants for resolution of inflammation is clearance of the inflammatory stimulus(23, 32). CGD is an example of where defects in clearance may be one of the primary causes of exacerbated and prolonged responses. Certainly, PMNs from CGD patients have impaired phagocytosis of immune-complexes while CGD macrophages are equally defective in their clearance of apoptotic PMNs(31). Equally, the formation of granulomatous synovitis in response to intra-articular zymosan injection in NADPH oxidase-deficient mice was suggested to result from incomplete zymosan clearance from the joint due to impaired phagocytosis

(26). This, therefore, suggests that dysregulation in cAMP and adenosine is secondary to that of an overwhelming inflammatory event, whose pro-inflammatory signals deactivates or overrides endogenous anti-inflammatory and/or pro-resolution pathways.

From the above argument it should not be assumed that all endogenous protective pathways are depressed during CGD. Among some of the signals that counter-balance inflammatory onset and/or trigger resolution, neither PGD_2 nor native lipoxin A_4 levels were statistically different in gp91phox$^{-/-}$ mice compared to wild types at onset with the exception of PGD_2, which showed a trend towards a reduction in knockouts as inflammation resolved. That notwithstanding, data presented here shows a more consistent dysregulation in the synthesis of adenosine/cAMP in gp91phox$^{-/-}$ mice in response to sterile zymosan. We went on to investigate and show that A_{2A} receptor activation rescues the hyper-inflammatory response in gp91phox$^{-/-}$ mice without subverting resolution in wild type animals. This latter point is important as existing anti-inflammatories, NSAIDS for instance, while being protective by virtue of their ability to dampen the early onset phase of acute inflammation, pirate resolution and prolong inflammation(22). A_{2A} receptor activation, on the other hand, is anti-inflammatory without being resolution-toxic thereby displaying broader pharmacological flexibility and potentially fewer side effects in terms of prolonging inflammation. However, these data are counter-intuitive based on current understanding of cAMP in inflammatory leukocyte longevity and clearance as derived from *in vitro* studies. For instance, elevating cAMP in PMNs delays their apoptosis(40) while raising cAMP in monocyte-derived macrophages impairs their phagocytic capacity(41) suggesting that activating A_{2A} during inflammation and consequently elevating cAMP would lengthen the life span of PMN, impair their clearance and prolong inflammation. Despite these data from isolated cell systems, *in vivo*-derived data from Figures 4 and 5 clearly show that A_{2A} receptor activation is anti-inflammatory without being resolution-toxic and that activation of this receptor at any phase of CGD, dampens inflammation. This implies that CGD is in a constant state of perpetual acute inflammation and that A_{2A} receptors inhibit PMN influx. The current treatment regime for CGD patients is antibacterial and antifungal prophylaxis(42), but for exacerbations of inflammatory events, perhaps concomitant A_{2A} receptor activation would dampen associated inflammatory responses without subverting pro-resolution pathways. At the very least, such patients should avoid caffeine (and perhaps other dietary methylxanthines such as theobromine) as it may nullify whatever protection residual adenosine may confer in CGD during inflammatory events.

As mentioned previously, being anti-inflammatory in pharmacological terms, is distinct from being pro-resolution(23). We make this assertion based on ongoing work from our laboratory where we have shown in the resolving peritoneum not only a disappearance of PMN *via* apoptosis and macrophages *via* lymphatic drainage, but the influx of innate-type lymphocytes as inflammation resolves(20). These repopulating lymphocytes do not switch off inflammation but modulate post-inflammatory responses to bacteria in the context of secondary infection. In fact, when the cellular composition of the naïve peritoneal cavity is examined, innate type lymphocytes are a predominant cell type along with resident macrophages. These lymphocytes disappear in response to inflammatory stimulus not before secreting cytokines that modulate the severity of the inflammatory response such that in RAG1$^{-/-}$ mice, for instance, inflammation is more exaggerated in terms of PMN trafficking. Therefore, as inflammation resolves, we suspect that repopulating lymphocytes simply reflect the inflamed tissue reverting to its prior physiological state under the control of as yet un-indentified endogenous factors. Interestingly, we found no repopulating lymphocytes in the peritoneal cavity of gp91phox$^{-/-}$ mice at the equivalent time points of resolution in wild types. Moreover, while activation of A_{2A} was certainly anti-inflammatory in gp91phox$^{-/-}$ mice by virtue of it inhibition of PMN numbers when administered therapeutically at the equivalent phase of resolution in wild types, it did not bring about resolution in gp91phox$^{-/-}$ as defined by its inability to trigger lymphocyte repopulation. Along these lines, activating A_{2A} with CGS 21680 at earlier time point, which

exerted classic anti-inflammatory effects as defined by reduced PMN numbers (Figure 4C and 5A-B) was also without effect on lymphocyte numbers. This suggests that other factors, besides that which signal cAMP are responsible for lymphocyte repopulation and reversion to homeostasis.

Our finding of adenosine being secreted and cAMP expressed during the early onset phase of the zymosan-induce peritonitis was not surprising given the established role these factors play in counter-regulating innate-immune mediated tissue damage(9,10). The re-appearance of cAMP and adenosine again at resolution, however, is a reflection of our growing understanding of resolution being an active, immuno-suppressive event controlled by endogenous counter-regulatory stop signals. The point however, is that adenosine/cAMP appears after the bulk of the inflammatory cells, including PMNs and monocyte-derived macrophages have disappeared either by apoptosis or lymphatic drainage. In another report (paper enclosed) we have identified the presence of a population of resolution-phase macrophages (rM) that have not vacated the peritoneum and are derived from a common Ly6C-positive monocyte precursor and which possess a unique phenotype. These rM cells express all the typical markers of alternatively-activated M2 cells along with iNOS and COX 2. It transpires that this phenotype is controlled by cAMP, which if inhibited or elevated transforms the phenotype of resolution-phase macrophage to that of M1 cells and *vice versa*, respectively. Thus, the post-resolution expression of cAMP is not required to switch off inflammation *per se* but is most likely the next step in post-inflammation tissue restitution and attempts to restore homeostasis.

From data presented in this study, A_{2A} is shown to be anti-inflammatory whilst not affecting pro-resolution pathways. However, it is well known that caffeine is a non-specific A_{2A} receptor antagonist as it can antagonise A_1 as well as A_{2A} but possesses a lower affinity for the A_3 receptor(39). Resulting from its non-specific inhibition of A_{2A}, caffeine may therefore worsen inflammation and negatively affect pro-resolution pathways. Indeed, dosing animals with 2mg/kg caffeine or its stable analogue just before zymosan injection exaggerated the inflammatory response 4h later and also impaired resolution. This is important as the amount of caffeine administered to mice is equivalent to a realistic 1-2 cups of coffee. Increasing doses of caffeine to unrealistic 10-30 mg/kg, as also done in this current study, caused a loss of caffeine's pro-inflammatory impact as at these levels and higher (100mg/kg) caffeine may become a PDE4 inhibitor resulting in cAMP elevation, as shown *in vivo* recently(43). Given the wide consumption of caffeine in the form of coffee and tea at least, we need to be aware of the data presented in this paper and that presented by others(43), which emphasises that interfering with endogenous protective pathways, adenosine in this case, at realistic levels of socially-consumed beverages will hamper innate immune responses, thus impairing the ability to combat infections concomitant with prolonging resolution. However, any attempts to increase caffeine intake in the hope of inhibiting PDE4 in order to dampen inflammation *via* cAMP elevation would require prohibitively high quantities of the drug. Thus, the most likely result of social caffeine consumption would be pro-inflammatory and resolution toxicity.

In conclusion, we show that of the endogenous anti-inflammatory pathways examined in CGD, both adenosine and its intracellular signalling molecule, cAMP, show dysregulation in their synthesis at onset and resolution suggesting that CGD is in a constant state of pro-inflammation and PMN trafficking with no apparent attempts at resolution due to the persistence of the inflammatory stimulus. Rescuing this hyper-inflammatory state with A_{2A} agonists shows powerful anti-inflammation that does not bring about resolution as it inhibits PMN trafficking but does not initiate lymphocyte repopulation and reversal to homeostasis. Nonetheless it does suggest a potential treatment regime to dampen the hyper-innate immune component of CGD-associated infections.

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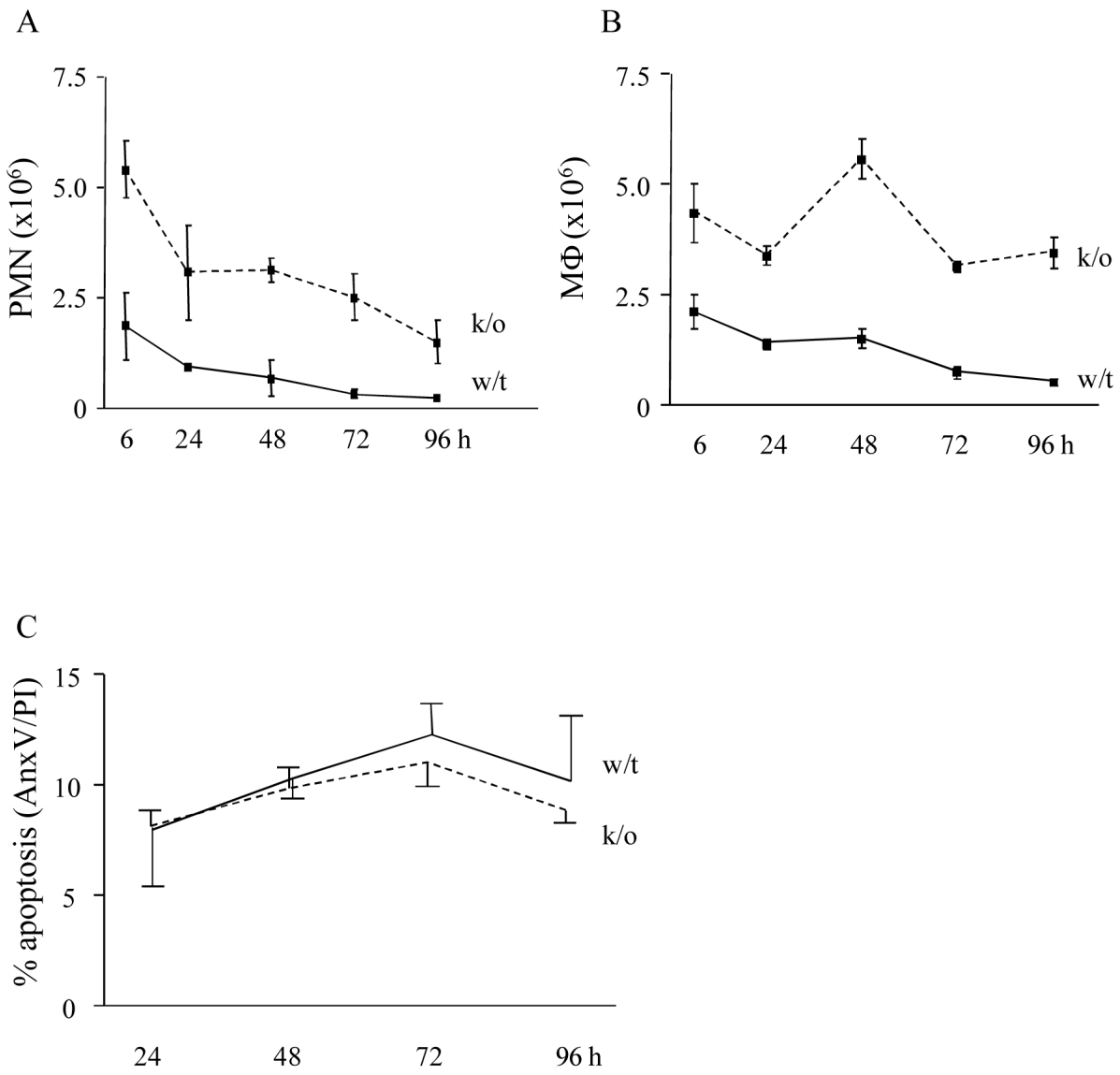

Figure 1.

Inflammation is more severe and fails to resolve in gp91phox$^{-/-}$ mice. Intraperitoneal zymosan injection resulted in a more exaggerated influx of (A) Ly6G-positive PMNs in gp91phox$^{-/-}$ mice along with (B) F4/80-positive monocyte-derived macrophages that persisted in these animals past the equivalent time point of resolution in wild types with the persistence of this response not due to (C) reduced apoptosis in gp91phox$^{-/-}$ mice. *n* = 8-10 animals per group with data expressed as mean ± SEM.

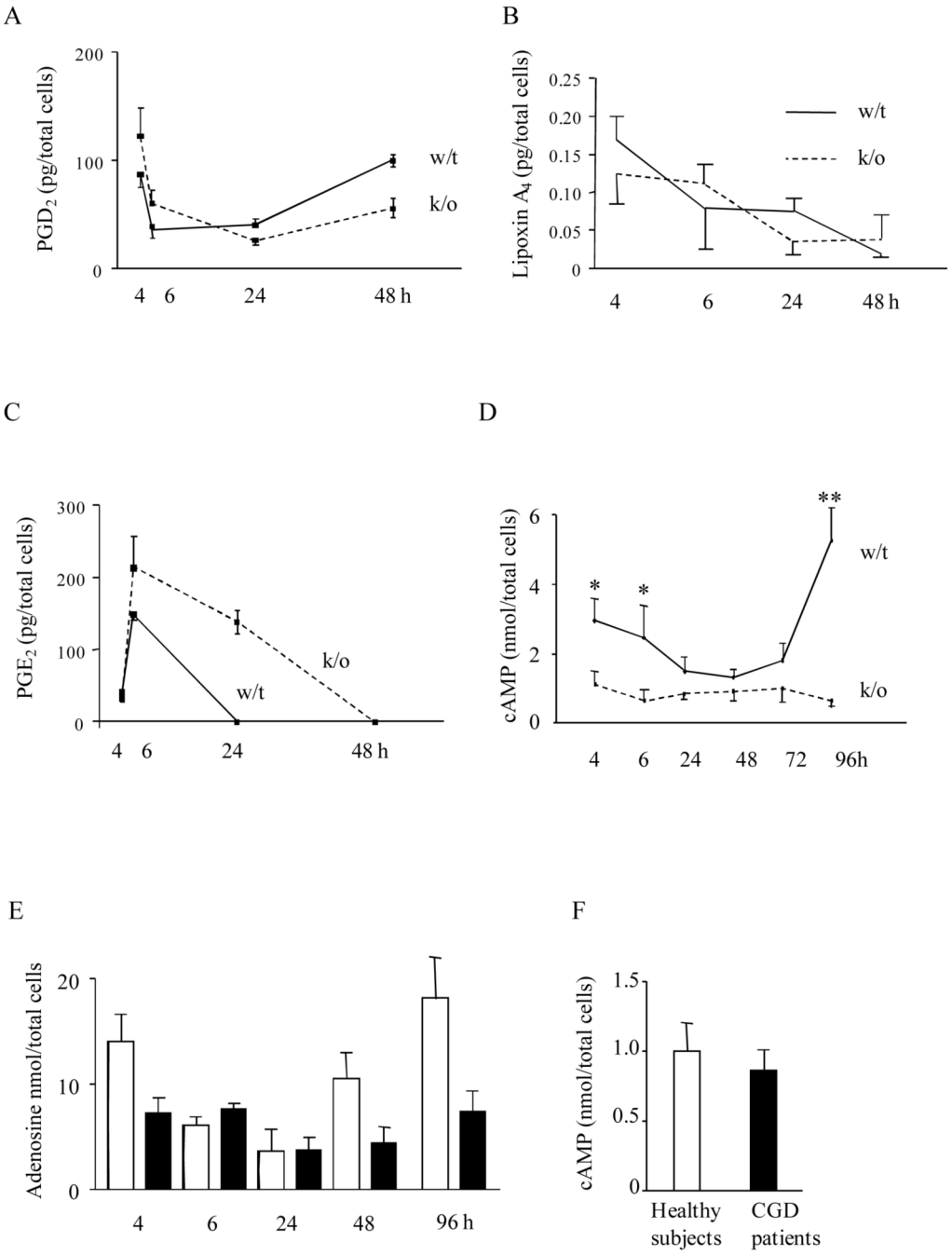

Figure 2.

Adenosine and cAMP synthesis is abrogated in gp91phox$^{-/-}$ mice. In an attempt to identify potential dysregulations in the synthesis of endogenous anti-inflammatory/pro-resolution factors in gp91phox$^{-/-}$ mice, arachidonic acid metabolism, as determined by (A) PGD_2, (B) lipoxin A_4 as well as (C) PGE_2 were determined and found not to show a consistent reduction in gp91phox$^{-/-}$ mice compared to wild types. However, (D) cAMP was elevated at the early onset phase of a murine peritonitis and then again at resolution in wild type animals, but was significantly lower in gp91phox$^{-/-}$ mice at both phases. (E) Adenosine, which signals through A2a receptors to elevate cAMP, was also measured in these peritonitis samples peaking at onset and again at resolution with levels being reduced in gp91phox$^{-/-}$ mice (filled columns)

compared to wild types (empty columns). While this would suggest dysregulated cAMP signalling in CGD, (F) release of cAMP from LPS-stimulated monocyte-derived macrophages obtained from healthy human volunteers was equivalent to that from CGD patients. This indicates that there is no direct interaction between NADPH oxidase and adenosine/cAMP, with the latter most likely being diminished or over ridden by suppressive factors released during severe inflammation typical of CGD. *n* = 6-10 animals per group with data expressed as mean ± SEM.

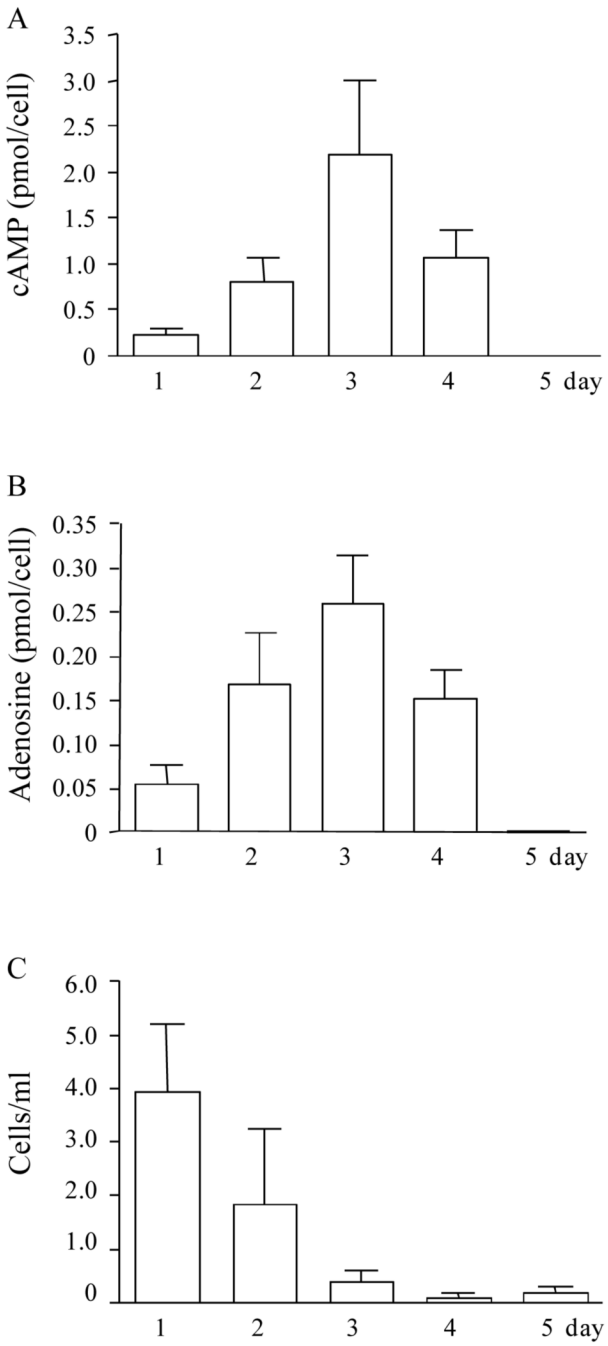

Figure 3.

Expression of adenosine and cAMP concomitant with resolution of human peritonitis. Peritonitis samples were obtained from patients undergoing chronic ambulatory peritoneal dialysis and who experience transient infection that usually resolves with a few days. Analysis of cell-free exudates revealed a peak in (A) cAMP and (B) adenosine as (C) inflammation resolved. n = 6-8 patients with data expressed as mean ± SEM.

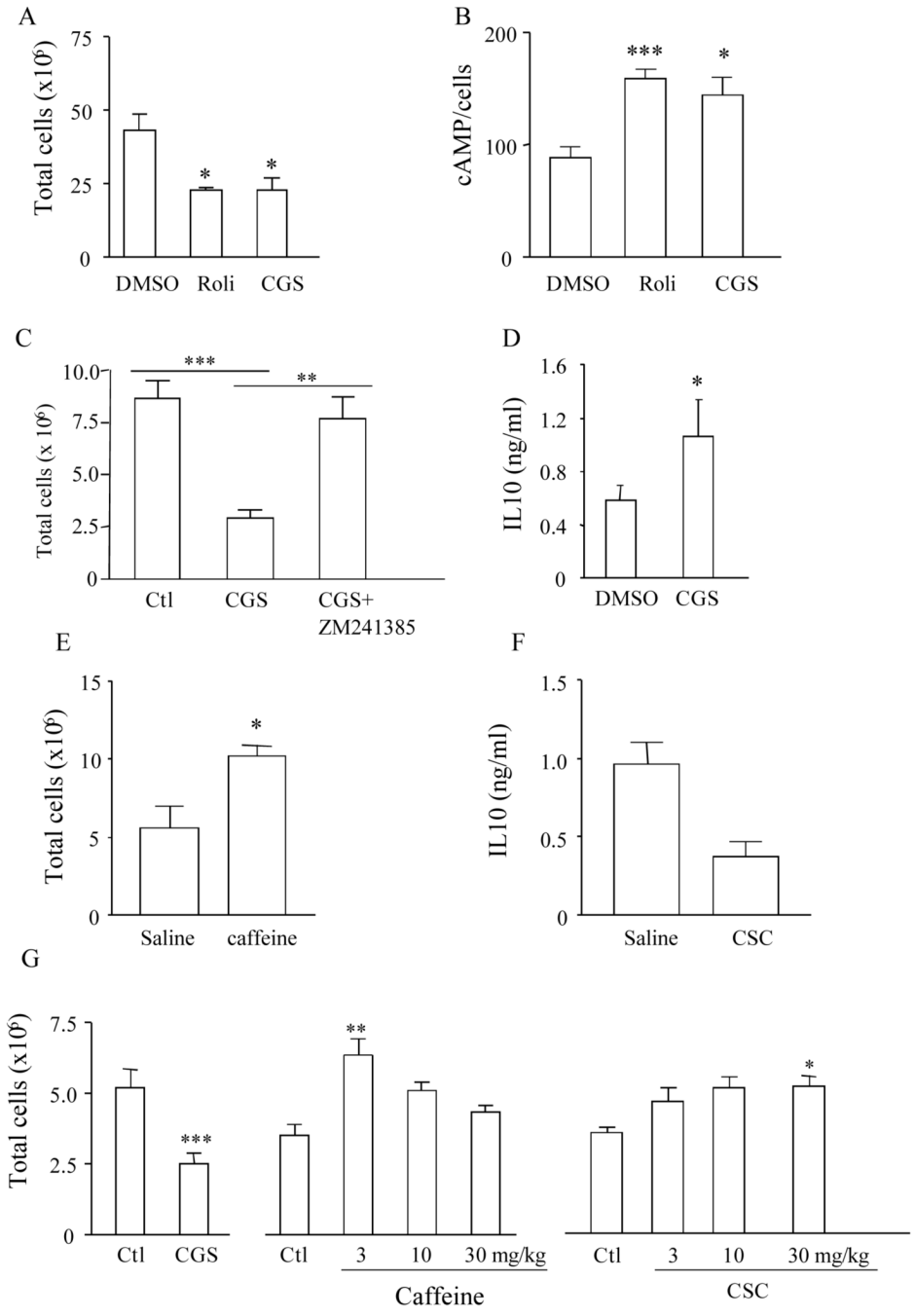

Figure 4.

Adenosine, *via* A_{2A} is anti-inflammatory and not resolution-toxic. The selective A_{2A} agonist CGS 21680 as well as the PDE4 inhibitor rolipram, which increases cAMP were dosed at 10 and 30 mg/kg respectively, to rats bearing a carrageenin-induced pleurisy. In (A) rolipram and CGS 21680 were dosed 30 mins prior to pleurisy induction and their effects assessed 4h later with A_{2A} receptor activation exerting an anti-inflammatory effect concomitant with an increase in (B) cAMP in this model. A_{2A} receptor activation was also examined in a zymosan-induced peritonitis dosed 30mins prior to zymosan injection and the effects of CGS 21680 with or without ZM241385 (A_{2A} receptor antagonist) determined 4h later revealing, again, that (C) CGS 21680 is anti-inflammatory in this model concomitant with an elevation in (D) anti-

inflammatory IL-10. Conversely, the A_{2A} receptor antagonist (E) caffeine and its (F) stable analogue, CSC worsened inflammation and depressed IL-10, respectively, when administered in a similar manner to receptor agonists. In the final set of experiments we demonstrated that A_{2A} receptor activation maintained its anti-inflammatory effects and did not interfere with resolution pathways as injection of (G) CGS 21680, given 30mins before zymosan and again 12h and 18h later continued to dampen inflammation while caffeine and its analogue worsened the response as determined at 24h. $n = 8$ animals per group; *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as mean ± SEM.

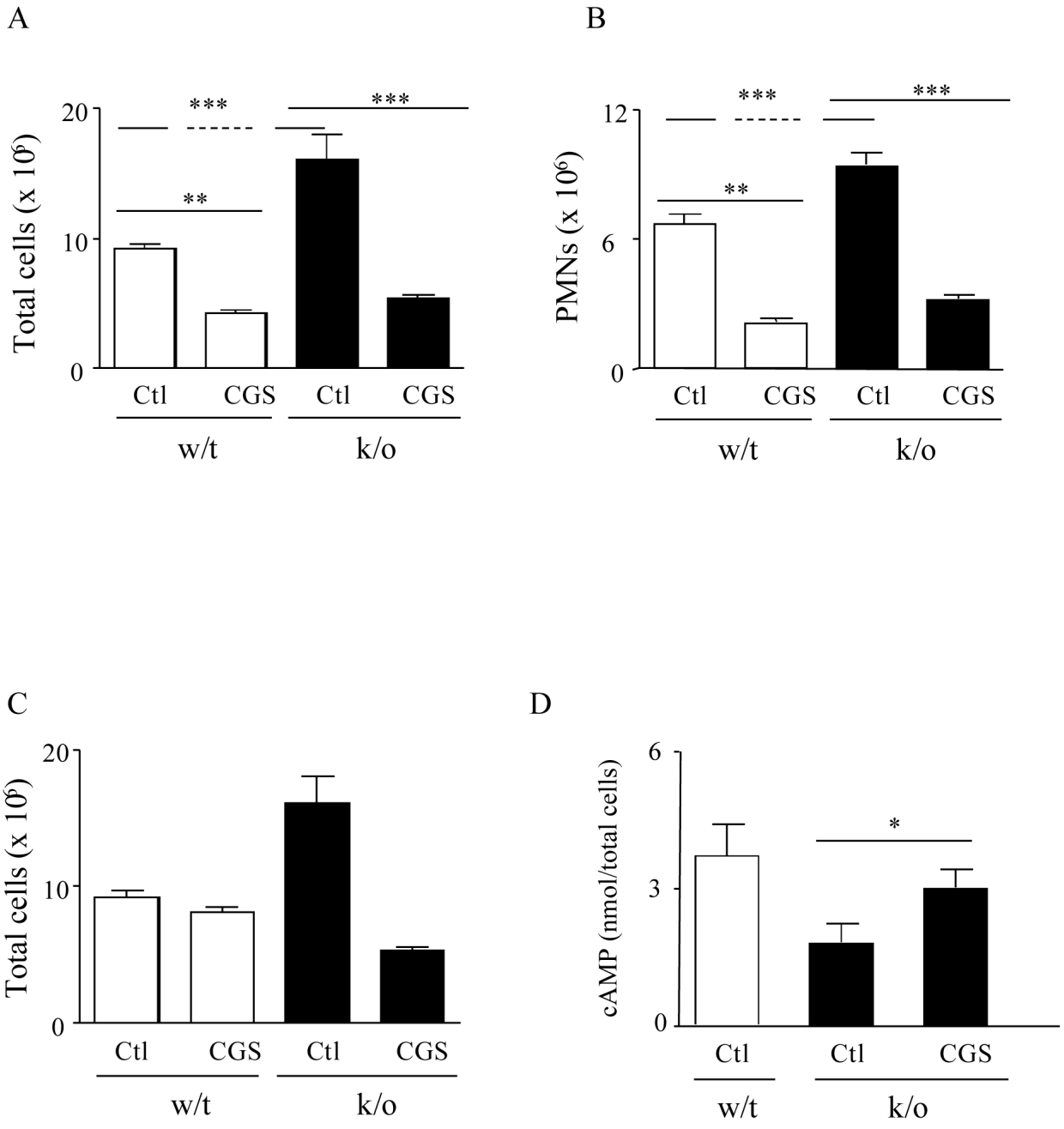

Figure 5.

Adenosine, *via* A_{2A} is anti-inflammatory but not pro-resolution in gp91phox$^{-/-}$ mice. Wild type and gp91phox$^{-/-}$ mice were injected with zymosan i.p. and the A_{2A} agonist CGS 21680 given either (A-B) 30mins before stimulus injection to both wild types and knockouts and its effects determined 4h later or (C) administered to both animal types therapeutically i.e. 24h and 36h after zymosan and its effects on resolution and (D) cAMP determined at 48h. $n = 6$ animals per group; *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as mean ± SEM.